

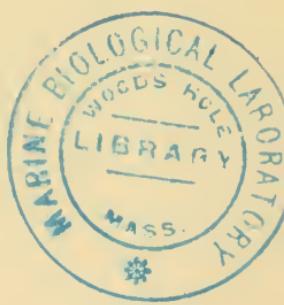




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MONOGRAPHS ON THE PROGRESS OF RESEARCH IN HOLLAND  
DURING THE WAR

MONOGRAPHS ON THE PROGRESS OF  
RESEARCH IN HOLLAND  
DURING THE WAR

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*Continued on page 147*

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SEXUAL  
ENDOCRINOLOGY OF  
NON-MAMMALIAN VERTEBRATES

by

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and

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*in collaboration with*

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Respectfully dedicated

*to*

PROFESSOR BERNHARD ZONDEK



## FOREWORD BY THE EDITORS

The purpose of this Series on the Progress of Research in Holland during the war is to show the world that scientists in the Netherlands have remained active during the five years of German occupation. The publication of monographs by the most representative research workers was already planned in the first years of the war, as a token of the undaunted spirit of the Netherlands.

In spite of the ever-growing burden of oppression and starvation research was continued intensively in all directions. Most of the material now published in this series was, for obvious reasons, kept a secret during the war.

It being the Editors' aim to present these monographs as early as possible after war, the majority of them were prepared whilst the war was still on. Authors were therefore expected to give mainly the results of their own investigations without exhaustive reference to the Anglo-Saxon literature which would not be available to them until after the war.

The hope is expressed that the publication of this series will further intensify the interest shown by the allied nations in the fate of the Netherlands, demonstrating, as it does, the whole-hearted preparedness of our nation to contribute to the progress of mankind.

The Editors,  
DR IR R. HOUWINK  
DR J. A. A. KETELAAR

Amsterdam, on V.E.-day, 8th May 1945.

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## P R E F A C E



On May 10, 1940, our country became a victim of German aggression. As far as circumstances permitted, the Working Community for Endocrinology, Utrecht, continued its activities during the troublesome years that followed, mainly thanks to the generous financial assistance received from the Technical Department of the Netherlands Central Organization for Applied Scientific Research; the JAN DEKKER Foundation, and the HECTOR TREUB Fund, to whom we wish to express our deep gratitude. We are also very grateful to Professor CHR. P. RAVEN for his unfailing interest in our research work, and to DR A. S. PARKES, F.R.S., Professor J. Z. YOUNG, Mrs. S. CARSWELL and Professor G. J. VAN OORDT, for reading the manuscript.



## CONTENTS

	Pag.
INTRODUCTION . . . . .	1
I THE FEMALE BITTERLING AS TEST OBJECT FOR ENDOCRINE EXPERIMENTS	4
1 THE OVIPOSITOR TEST . . . . .	4
2 THE SPECIFICITY OF THE OVIPOSITOR TEST . . . . .	6
3 THE REACTION TO STEROID HORMONES . . . . .	9
A <i>The reaction to estranes</i> . . . . .	9
B <i>The reaction to pregnanes</i> . . . . .	10
C <i>The reaction to androstanes</i> . . . . .	18
4 THE REACTION TO GLANDULAR EXTRACTS . . . . .	26
5 THE REACTION TO URINE . . . . .	34
6 SUMMARY . . . . .	38
7 THE LUTEIDIN PROBLEM . . . . .	39
II THE CONCATENATION HYPOPHYSIS→ OVARY→OVIPOSITOR IN THE BITTER- LING . . . . .	46
1 THE HISTO-PHYSIOLOGICAL EXAMINATION OF THE HYPOPHYSIS . . . . .	46
2 HOMOLOGY OF THE HYPOPHYSIS-LOBES . . . . .	56
3 THE BLOOD SUPPLY OF THE HYPOPHYSIS IN <i>Rhodeus amarus</i> . . . . .	62
4 THE INNERVATION OF THE BITTERLING HYPO- PHYSIS . . . . .	65
5 THE HAEMO- AND NEUROCRINIA OF THE HYPO- PHYSIS OF THE BITTERLING . . . . .	69
6 SUMMARY . . . . .	73

III THE CORPUS LUTEUM AND OTHER FOLLICULAR DERIVATIVES IN VERTE- BRATES . . . . .	77
1 THE STRUCTURE OF THE OVARY . . . . .	77
2 HISTOGENESIS OF THE CORPORA LUTEA . . . . .	81
3 THE OVO-FOLLICULAR SYSTEM . . . . .	93
4 THE POTENTIALITIES OF THE FOLLICLE . . . . .	96
5 SERIAL REPETITION OF THE POSSIBLE MODIF- ICATIONS IN THE FOLLICLE . . . . .	101
6 THE DERIVATIVES OF FOLLICULAR INVOLUTION AND THE INTERSTITIUM . . . . .	108
7 THE SIGNIFICANCE OF THE CORPORA LUTEA AND OTHER FOLLICULAR DERIVATIVES IN THE SEXUAL-ENDOCRINE ORGANIZATION OF VERTE- BRATES . . . . .	110
8 THE FREQUENCY OF THE FOLLICULAR DERIVAT- IVES IN THE DIFFERENT GROUPS OF VERTE- BRATES . . . . .	113
9 SUMMARY . . . . .	116
IV THE CYCLIC CHANGES IN THE SEXUAL- ENDOCRINE ORGANIZATION OF NON- MAMMALIAN VERTEBRATES . . . . .	117
1 THE SEXUAL CYCLE OF <i>Rhodeus amarus</i> ♀ . . . . .	117
2 THE SEXUAL CYCLE OF <i>Zoarces viviparus</i> ♀ . . . . .	126
3 THE SEXUAL CYCLE OF <i>Lebistes reticulatus</i> ♀ . . . . .	129
4 THE SEXUAL CYCLE OF <i>Bufo bufo</i> ♂ AND ♀ . . . . .	134
5 SUMMARY . . . . .	140
BIBLIOGRAPHY . . . . .	143
INDEX . . . . .	145

## INTRODUCTION



BROWN-SEQUARD, the first to prepare testis extracts, used them on himself for the treatment of senility, and endocrinology owes much of its rapid development to anthropocentric interest. Most of the work, however, has been carried out on the smaller mammals, which are so much more accessible than man, and in more recent years endocrine processes in non-mammalian vertebrates have also been increasingly studied. This last branch of investigation is still in its initial stages.

In 1935 the present authors began to investigate the sexual-endocrine organization of non-mammalian vertebrates, and this led to the establishment, in 1939, of the "Werkgemeenschap voor Endocrinologie" (Working Community for Endocrinology) at Utrecht, under the auspices of the late Professor H. J. JORDAN and Professor CHR. P. RAVEN. The purpose of the present work is to summarize the material dealt with and the work done during the war-years 1940—1945. Where necessary, reference is made to previous work.

One difficulty met with when investigating the sexual-endocrine organization of non-mammalian vertebrates is that the phenomena to be studied are far less conspicuous than in mammals. On the other hand, the smaller dimensions and greater simplicity of structure of the organs are advantages.

It would be unsafe, however, to draw conclusions, in regard to possible analogies, from similar phenomena observed in mammals and non-mammals. On closer examination it may appear that such phenomena show quite essential differences. A typical example of this is given below.

FLEISCHMANN and KAHN (1932) must be given credit for introducing the female bitterling (*Rhodeus amarus*, BLOCH) as a test animal for the study of sexual endocrinology. The bitterling is a Cyprinid fish, found fairly generally all over the Western part of continental Europe. In spawning time,

it develops a plainly visible urogenital papilla, the ovipositor, with which it lays its eggs in freshwater mussels of the genera *Unio* and *Anodonta*. Before and after spawning this tube shrinks to a hardly visible nipple; but FLEISCHMANN and KAHN found that the ovipositor can develop at any time into a long tube when estrogenic hormone is added to the aquarium water. Struck by the similarity between this phenomenon and the changes occurring in the mammalian uterus during the rutting season, they concluded that the estrogenic hormone in mammals must also be a physiological estrogenic hormone in the fish. From our experiments, however, it has become clear that steroid mammalian hormones affect the ovipositor indirectly, and that their influence on the bitterling is probably pharmacological!

We realized this when BRETSCHNEIDER observed the presence of numerous *corpora lutea* in the ovary of the bitterling at the time of the growth of the ovipositor. He further correlated the occurrence of these corpora lutea with the presence of *basophilic cells* in the so-called gonadotrophic zone of the anterior pituitary lobe. These basophils were regarded as the restitution phase of the cells producing the gonadotrophic hormone. After injection of an extract of the hypophysis of the carp there was marked formation of corpora lutea. When the hypophysis was removed, administration of steroid hormones did not result in growth of the ovipositor. Again, after ovarieotomy, steroid hormones did not cause growth of the ovipositor when the hypophysis was left intact, whereas an extract from the ovary of *Lophius piscatorius* containing corpora lutea caused appreciable growth of the ovipositor in intact fishes. It seems, therefore, that the steroid (mammalian) hormones influence the anterior pituitary lobe (? via the hypothalamus), resulting in the secretion of gonadotrophic hormone and the development of numerous corpora lutea, whose hormone induces growth of the ovipositor.

Because of its ovipositor the female bitterling is well suited to the investigation we had in view. Hence we subjected the sexual-endocrine organization of this fish to an exhaustive

analysis. The observations we made have not only enriched our knowledge of the sexual-endocrine processes in amphibia, reptiles, and birds, but have also given us a clearer understanding of similar processes in mammals.



## I. THE FEMALE BITTERLING AS TEST OBJECT FOR ENDOCRINE EXPERIMENTS

### § I. THE OVIPOSITOR TEST

FLEISCHMANN and KAHN (1932), EHRHARDT and KUHN (1933), KANTER, BAUER, and KLAWANS (1934) have employed the ovipositor test in hormone experiments. We have elaborated their method into a quantitative one.

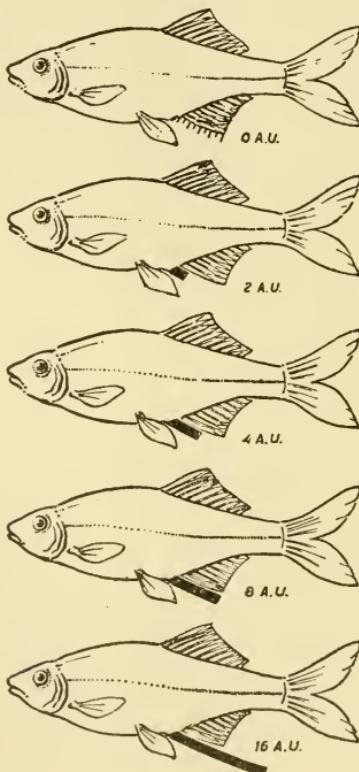


Fig. 1. Diagram representing the female bitterling with anal finray divided into 8 parts showing the length in A.U. (anal fin units) of the ovipositor.

The ovipositor is measured in so-called "anal fin units" (A.U.). One A.U. corresponds to one-eighth part of the foremost radius of the extended anal fin (Fig. 1). We use small aquaria of 750 ml capacity, in which there are three fishes, distinguishable from each other by their respective sizes. These small aquaria are placed inside a larger one serving as thermostat, and in which an optimal temperature of 22° C is maintained (Fig. 2). The small aquaria are well aerated.

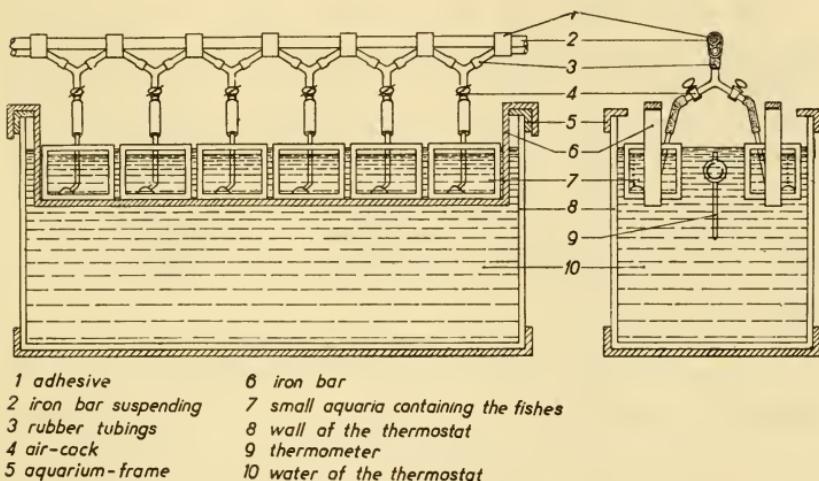


Fig. 2. Front and side views of the testing equipment.

When measurements are taken (every hour or two), the small aquaria are removed from the thermostat and held up to the light for a few moments, when it is possible to estimate the length in A.U. of the ovipositor by eye and with sufficient accuracy.

When new fishes are used, and transferred from the cold water to that at 22° C, there is an autonomous growth of a few A.U. The first reaction to a steroid hormone, moreover, is generally too low. For this reason we first sensitize the fishes by causing them to react to the urine of pregnant women (3 ml per 750 ml water). After 12 to 14 hours the ovipositors of the majority of the fishes have grown markedly. Fishes that have reacted insufficiently are eliminated. The

others are put in clean water. After 24 hours the ovipositors have shortened to a length of from 2 to 4 A.U., and are most ready to react.

When it is desired to test a given preparation, it is advisable to get a number of the fishes, for control purposes, to react to a standard preparation, say progesterone (5-8  $\gamma$  per 750 ml water). In this way it is possible to compare the results of different days' tests, as the sensitivity of the fishes fluctuates.

The fishes may be used several times in succession, providing their ovipositors have an initial length of between 2 and 4 A.U. for each test. Insufficient reaction to the standard preparation gives warning that the fishes are to be considered as finished with, and to be replaced by fresh ones.

The preparations need not be injected; they should simply be added to the water. Anterior-pituitary preparations, however, do not permeate the epithelium of the gills, and must therefore be injected.

It is usually sufficient for exact determination to obtain an average reaction of 60 fishes. The activity of the preparations is expressed in the curve of the (average) growth of the ovipositors. The fishes should be properly fed, on a diet for omnivorous fishes, since a great deal is expected of them physiologically.

It is essential that the aquaria should be properly and constantly lighted, since pituitary activity is dependent on light. The reaction of the ovipositor is favourably affected by permanent electric light (20—25 % higher results).

## § 2. THE SPECIFICITY OF THE OVIPOSITOR TEST

We have mentioned that the female bitterling reacts to the steroids of the estrane, pregnane, and androstane range. Although the fishes are most sensitive to these, they also react to various other substances of quite a different chemical constitution. The specificity of the ovipositor test, therefore, is such that one should always take into account what other

substances may be present in the preparation to be tested, and especially the solvent itself.

According to DUYVENÉ DE WIT, *the following substances were inactive*:-

Yohimbine	2	mg per	750	ml water
Cholesterol	5	"	750	"
Digoxigenin	6	"	750	"
Ergosterol	10	"	750	"
Vitamin D <sub>2</sub>	2	"	750	"

According to VAN KOERSVELD, *the following substances were inactive*:-

Galactose	200	"	750	"	"
Maltose	200	"	750	"	"
Saccharose	200	"	750	"	"
Glycogen	200	"	750	"	"
Gelatin	200	"	750	"	"
Casein	200	"	750	"	"
Glycine	200	"	750	"	"
Ascorbic acid	250	"	750	"	"
Glucose	22½ g	"	750	"	"
α-tocopherol acetate	8 mg	"	750	"	"

*The following were active*:-

Ethanol	2	ml per	750	ml water	gave 1	A.U. after 7 h
Butanol	0.5	"	750	"	1.4	" 7 "
Veratryl-alcohol	0.05	"	750	"	1	" 7 "
Glycerol	20	"	750	"	1.2	" 7 "
Glycocholic acid	50 mg	"	750	"	2.1	" 16 "
Desoxycholic acid	50	"	750	"	2.2	" 16 "
Sodium-taurocholate	100	"	750	"	4.6	" 16 "
Taurin	50	"	750	"	0.9	" 16 "

One should, therefore, take the greatest care when selecting the solvent. We generally use ethanol, which does not interfere significantly with the reaction when administered in a maximal quantity of 0.5 ml per 750 ml water. Propylene glycol, also a suitable solvent for steroid hormones is, considerably less active than ethanol, and is therefore useful. Veratryl-

alcohol, however, used as solvent for  $\alpha$ -tocopherol acetate, disturbs the reaction in a concentration of 0.05 ml per 750 ml water. For this reason we thought that vitamin E must be active in the ovipositor test (DUYVENÉ DE WIT, 1941). This, however, proved to be incorrect (VAN KOERSVELD). Vitamin E, added in solution to the aquarium water, is inactive. We have not yet tested its activity when injected; nor have we investigated its influence upon the fertility of fishes.

Closer consideration of the reaction to members of the estrane, pregnane, and androstane group shows that the growth-curves obtained with these substances differ, which again proves the specificity of the ovipositor test. This is illustrated in Fig. 3, where the growth-curves, obtained after ad-

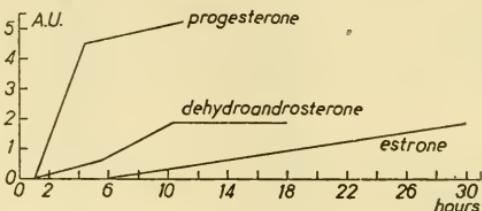


Fig. 3. Ovipositor growth-curves after the administration of progesterone, dehydro-androsterone and estrone. Note the typical differences between the respective curves.

ministration of optimal quantities of progesterone, dehydro-androsterone and estrone, respectively, are given. It will be seen that progesterone shows a latent period of 1 hour; after 4½ hours the period of linear growth is ended. With dehydroandrosterone, the latent period is also 1 hour; but 5½ hours after the commencement of the experiment, linear growth increases in intensity. With estrone the latent period is 5½ hours, after which a linear growth period sets in, up to 60 hours after the commencement.

Although these values are, in the absolute senses, subject to seasonal fluctuations, their relative differences remain for the greater part unchanged.

On the basis of these differences it is possible to differentiate between the representatives of the estrone, pregnane, and

androstane groups. As will appear from the next paragraph, the growth- and concentration-curves of the respective members also differ from each other, but to a lesser extent.

### § 3. THE REACTION TO STEROID HORMONES

#### A. *The reaction to estranes.*

a. *Estrone.* With concentrations of 20, 250 and 1500  $\gamma$  per 750 ml water the following growth-curves were obtained (Fig. 4). The characteristics of these curves are:—

Latent period, 5  $\frac{1}{2}$  hours. Growth ceases after 60 hours.

b. *Estradiol.* With concentrations of 12  $\frac{1}{2}$ , 25, 250 and 500  $\gamma$  per 750 ml water the following growth-curves were obtained (Fig. 5). The characteristics of these curves are:—

Latent period, 5  $\frac{1}{2}$  hours. Growth ceases after 60 hours.

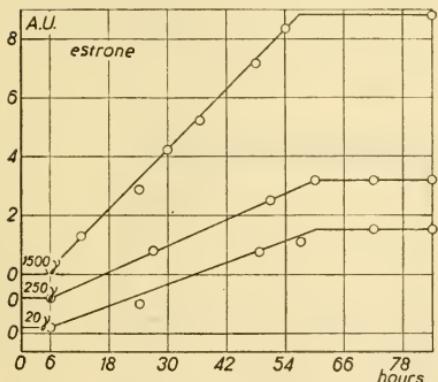


Fig. 4. Ovipositor growth curves after administration of estrone.

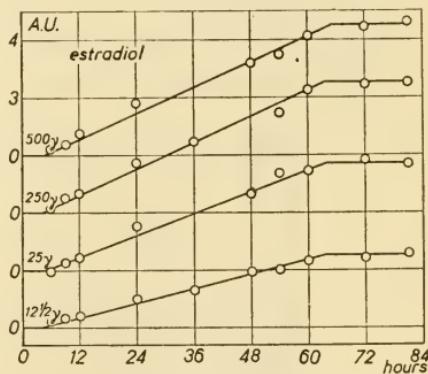


Fig. 5. Ovipositor growth curves after administration of estradiol.

c. *Estriol.* With concentrations of 25, 500, 1000 and 1500  $\gamma$  per 750 ml water the following growth-curves were obtained (Fig. 6). The characteristics of these curves are:—

Latent period, 5  $\frac{1}{2}$  hours. Growth ceases after 48 hours.

d. *Equilenin.* With a concentration of 1250  $\gamma$  per 750 ml water the following growth-curve was obtained (Fig. 7). The characteristics of this growth-curve are:—

Latent period, 5  $\frac{1}{2}$  hours. After 30 hours the growth-curve still continues.

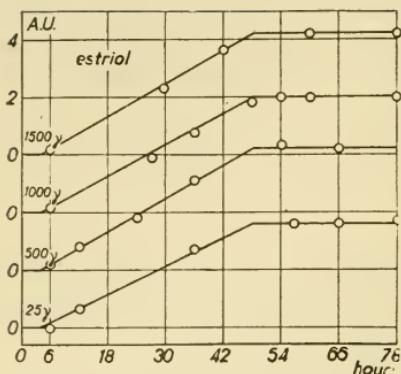


Fig. 6. Ovipositor growth curves after administration of estriol.

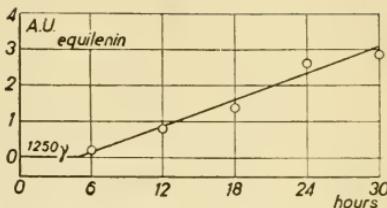


Fig. 7. Ovipositor growth curves after administration of equilenin.

### B. The reaction to pregnanes.

a. Progesterone (4,5-pregnenedione, 3-20). With concentrations of 4, 6, 7½, 10, 15, 20, 25 and 30 γ per 750 ml water the following growth-curves were obtained (Fig. 8). The characteristics of these curves are:—

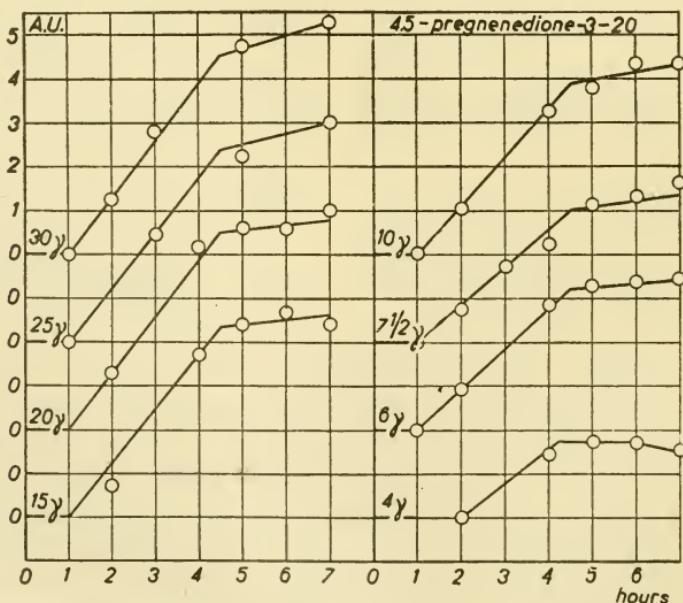


Fig. 8. Ovipositor growth curves after administration of 4,5-pregnenedione, 3-20.

Latent period, 1 hour. Linear growth ends after  $4\frac{1}{2}$  hours. Growth continues to some extent after this.

b. *Allo-pregnanedione, 3-20.* With concentrations of 5, 10, 20, 50, 100 and 150  $\gamma$  per 750 ml water the following growth-curves were obtained (Fig. 9). The characteristics of these curves are:—

Latent period, 1 hour. Linear growth ends after 4 hours.

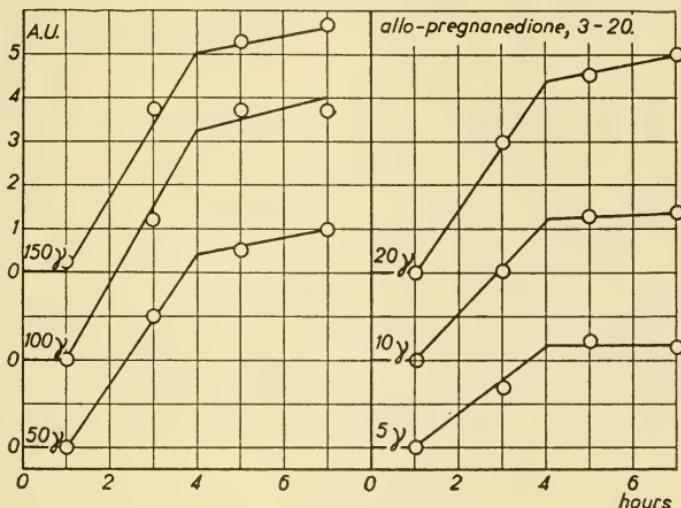


Fig. 9. Ovipositor growth curves after administration of allo-pregnanedione, 3-20.

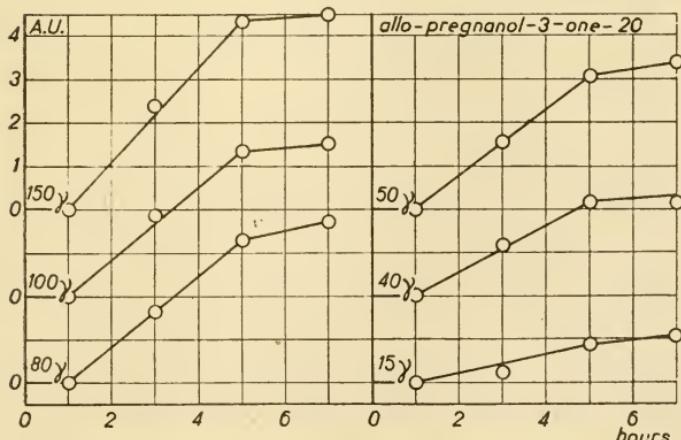


Fig. 10. Ovipositor growth curves after administration of allo-pregnano-3-one-20.

c. *Allo-pregnanol, 3-one-20.* With concentrations of 15, 40, 50, 80 and 100  $\gamma$  per 750 ml water the following growth-curves were obtained (Fig. 10). The characteristics of these curves are:—

Latent period, 1 hour. Linear growth ends after 5 hours.

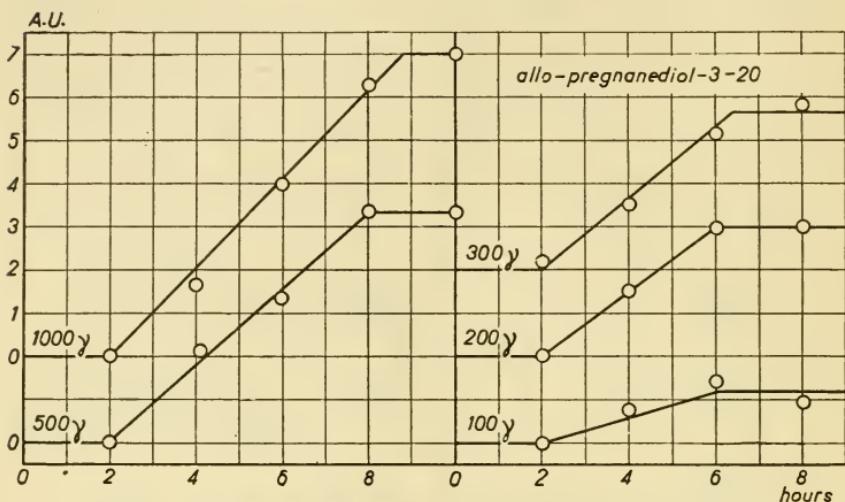


Fig. 11. Ovipositor growth curves after administration of allo-pregnenediol, 3-20.

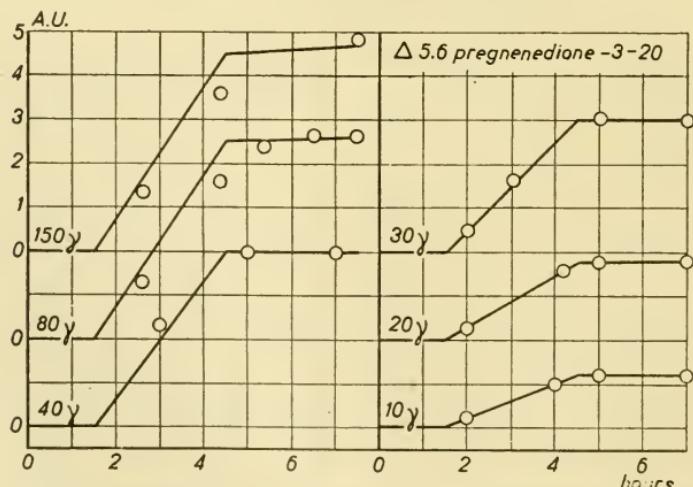


Fig. 12. Ovipositor growth curves after administration of  $\Delta$  5,6 pregnenedione, 3-20.

d. *Allo-pregnandiol, 3-20.* With concentrations of 100, 200, 300, 500 and 1000  $\gamma$  per 750 ml water the following growth-curves were obtained (Fig. 11). The characteristics of these curves are:—

Latent period, 2 hours. Linear growth ends after 8 hours.

e.  $\Delta$  5,6 pregnenedione, 3-20. With concentrations of 10, 20, 30, 40, 80 and 150  $\gamma$  per 750 ml water the following growth-curves were obtained (Fig. 12). The characteristics of these curves are:—

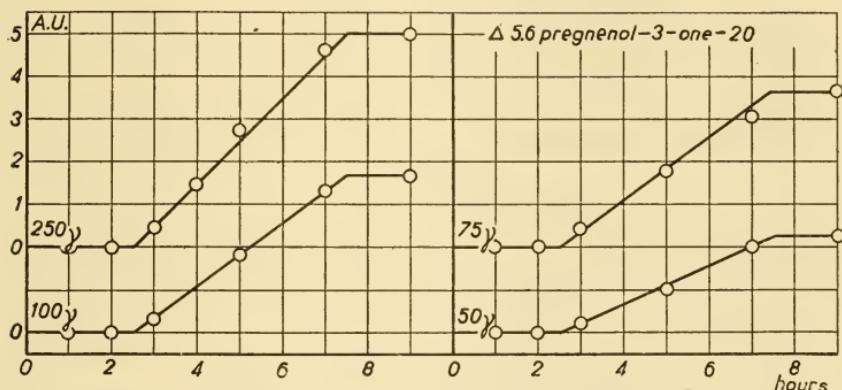


Fig. 13. Ovipositor growth curves after administration of  $\Delta$  5,6 pregnenol-3-one-20.

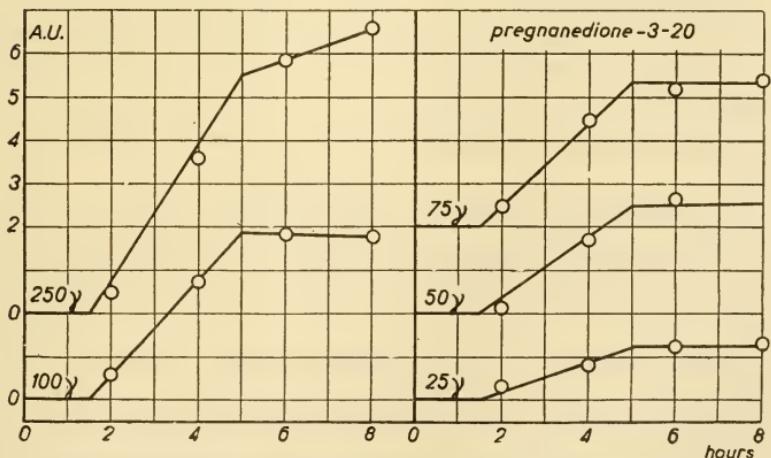


Fig. 14. Ovipositor growth curves after administration of pregnanedione, 3-20.

Latent period,  $1\frac{1}{2}$  hours. After this the curve runs horizontally.

f.  $\Delta$  5,6 pregnenol, 3-one-20. With concentrations of 50, 75, 100 and 250  $\gamma$  per 750 ml water the following growth-curves were obtained (Fig. 13). The characteristics of these curves are:—

Latent period,  $2\frac{1}{2}$  hours. Linear growth ends after  $7\frac{1}{2}$  hours.

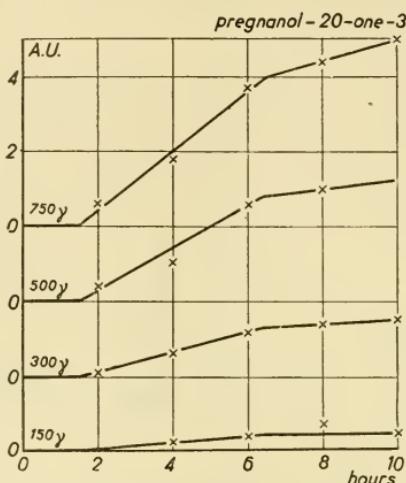


Fig. 15. Ovipositor growth curves after administration of pregnanol-20-one-3.

g. Pregnanedione, 3-20. With concentrations of 25, 50, 75, 100 and 250  $\gamma$  per 750 ml water the following growth-curves were obtained (Fig. 14). The characteristics of these curves are:—

Latent period,  $1\frac{1}{2}$  hours. Linear growth ended after 5 hours.

h. Pregnanol, 20-one-3. With concentrations of 150, 300, 500 and 750  $\gamma$  per 750 ml water the following growth-curves were obtained (Fig. 15). The characteristics of these curves are:—

Latent period,  $1\frac{1}{2}$  hours. Linear growth ends after  $6\frac{1}{2}$  hours.

i. Pregnanediol, 3-20. Only with the high concentration of 2000  $\gamma$  per 750 ml water could a distinct reaction be obtained (Fig. 16). The growth-curve is probably of the type

characteristic of male hormones. The characteristics of this curves are:—

Latent period, 1 hour. Linear growth, during the first phase, lasts at least  $5\frac{1}{2}$  hours.

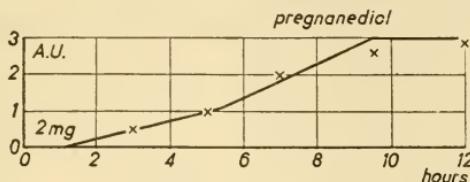


Fig. 16. Ovipositor growth curves after administration of pregnanediol.

It is interesting to consider more closely the relation between the biological activity and the chemical constitution of the 9 substances mentioned above.

We shall define the most active hormone as that of which, irrespective of the duration of the latent period, the smallest quantity per unit of time produces the greatest initial growth of the ovipositor. The activity may then be further defined as the amount of growth per hour caused by 1  $\gamma$  of hormone per 750 ml water at 22° C, providing that the hormonal concentration does not change significantly after the reaction ceases.

In the case of pregnenes, pregnanes, and allo-pregnanes, the diones are always more active than the olones. In their turn the olones are always more active than the dioles.

It further appears that, of the diones,  $\Delta$  4,5-pregnene is nearly 4 times as active as  $\Delta$  5,6-pregnene, and allo-pregnane 9 times as active as pregnane.

Of the olones, allo-pregnane is 14 times as active as pregnane, and nearly  $1\frac{1}{2}$  times as active as  $\Delta$  5,6-pregnene.

Of the dioles, allo-pregnane is 36 times as active as pregnane.

The activity of the allo-pregnanes evidently depends on the number of = O-groups. When one = O is replaced by one -OH, activity is weakened 8 times. When a second = O is replaced by -OH, there is again a 4-fold weakening.

The activity of the pregnanes appears to be dependent on

the number of = O-groups. When one = O is replaced by one -OH-group, the activity is weakened 13 times. When the second = O is replaced by an -OH, there is again a weakening of from 5 to 10 times.

The duration of activity, in the case of the allo-pregnanes and the pregnanes, also depends on the number of = O or -OH-groups. When one -OH is replaced by an = O, the duration of the activity is shortened about  $1\frac{1}{2}$  times. When a second -OH is replaced by an = O, the duration of the activity is shortened again  $1\frac{1}{2}$  times.

It is relevant at this point to deal with the reactions of some of the corticosterone-derivatives.

a. *Corticosterone*. With concentrations of 200, 400, 600 and 750  $\gamma$  per 750 ml water, the following growth-curves were obtained (Fig. 17). The characteristics of these curves are:—

Latent period, 1 hour. Linear growth ends after  $4\frac{1}{2}$  hours. After this the growth-curve remains normal.

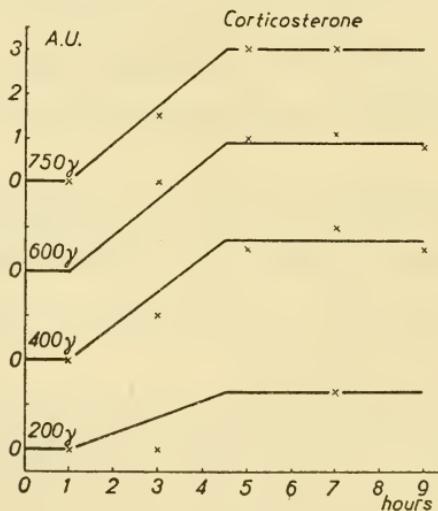


Fig. 17. Ovipositor growth curves after administration of corticosterone.

b. *Desoxycorticosterone*. With concentrations of  $2\frac{1}{2}$ , 4, 5,  $7\frac{1}{2}$ , 10, 15, 20 and 60  $\gamma$  per 750 ml water the following growth-curves were obtained (Fig. 18). The characteristics of these curves are:—

Latent period, 1 hour. Linear growth ends after  $4\frac{1}{2}$  hours. After this the curve runs horizontally.

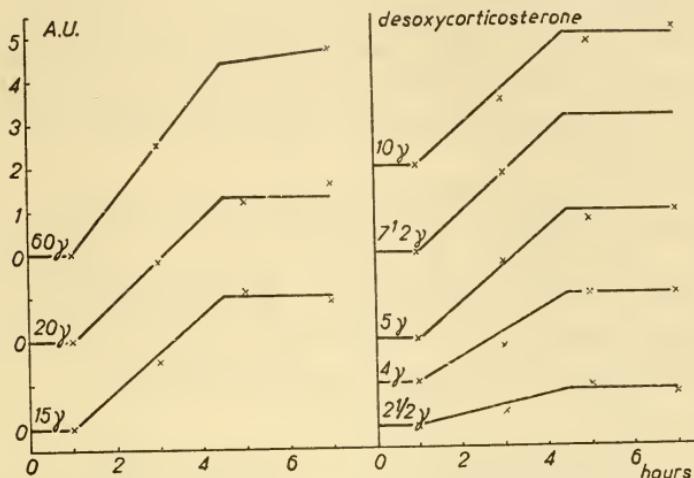


Fig. 18. Ovipositor growth curves after administration of desoxycorticosterone.

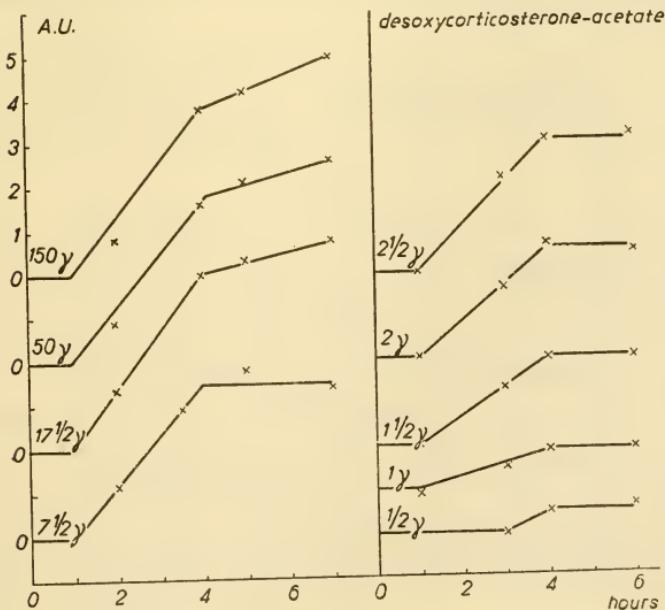


Fig. 19. Ovipositor growth curves after administration of desoxycorticosterone-acetate.

c. *Desoxycorticosterone-acetate*. With concentrations of  $\frac{1}{2}$ , 1,  $1\frac{1}{2}$ , 2,  $2\frac{1}{2}$ ,  $7\frac{1}{2}$ ,  $17\frac{1}{2}$ , 50 and 150  $\gamma$  per 750 ml water the following growth-curves were obtained (Fig. 19). The characteristics of these curves are:—

- 1 Latent period, 1 hour. Linear growth ends after 4 hours.
- 2 It appears from the above that desoxycorticosterone is 80 times, and desoxycorticosterone-acetate 200 times as active as corticosterone.

It is interesting that corticosterone  $\Delta$  4,5 pregnene, 11, 21-diole-3,20-dione) is 60 times less active than progesterone, while desoxycorticosterone (21-oxy-progesterone) is 1,3 times as active as progesterone. Perhaps the activity of a pregnene is weakened by an OH-group, attached to C<sub>11</sub>, but intensified by an OH-group, attached to C<sub>21</sub>.

### C. The reaction to androstanes.

a.  $\Delta$  4,5-androstanedione-3,17. With concentrations of  $\frac{1}{4}$ ,  $\frac{1}{2}$ , 1 and  $1\frac{3}{4}$  mg per 750 ml water the following growth-curves were obtained (Fig. 20). The characteristics of these curves are:—

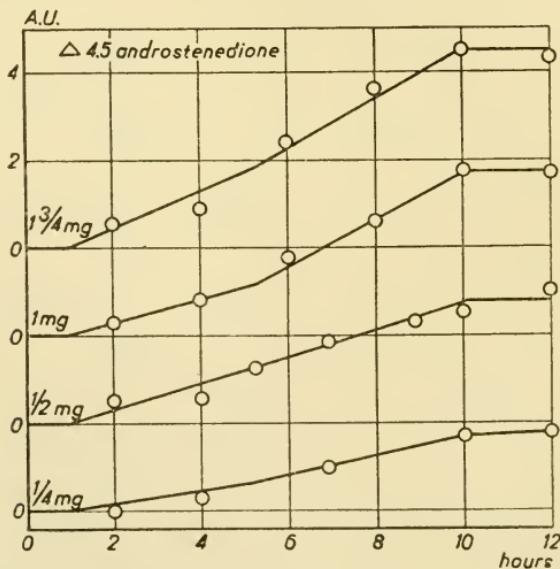


Fig. 20. Ovipositor growth curves after administration of  $\Delta$  4,5 androstanedione.

Latent period, 1 hour. Growth ceases after 10 hours.

b.  $\Delta 4,5$ -androstenol-17-trans-one-3 (*trans*-testosterone). With concentrations of  $\frac{1}{8}$ ,  $\frac{1}{4}$ ,  $\frac{1}{2}$ , 1 and 2 mg per 750 ml water the following growth-curves were obtained (Fig. 21). The characteristics of these curves are:—

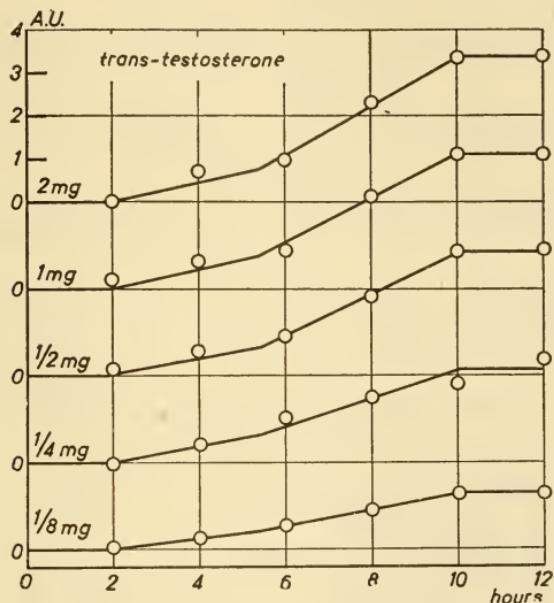


Fig. 21. Ovipositor growth curves after administration of *trans*-testosterone.

Latent period, 2 hours. Growth ceases after 10 hours.

c.  $\Delta 5,6$ -androstenol-3-trans-one-17 (*trans*-dehydroandrosterone). With concentrations of  $\frac{1}{4}$ ,  $\frac{1}{2}$ , 1 and 2 mg per 750 ml water the following growth-curves were obtained (Fig. 22). The characteristics of these curves are:—

Latent period, 1 hour. With higher concentration growth continues for more than 12 hours.

d.  $\Delta 5,6$ -androstene-3-trans, 17-trans-diol. With concentrations of  $20\gamma$ ,  $\frac{1}{16}$ ,  $\frac{1}{8}$ ,  $\frac{1}{4}$ ,  $\frac{1}{2}$  and 1 mg per 750 ml water the following growth-curves were obtained (Fig. 23). The characteristics of these curves are:—

Latent period, 1 hour. With higher concentrations, growth continues after 12 hours.

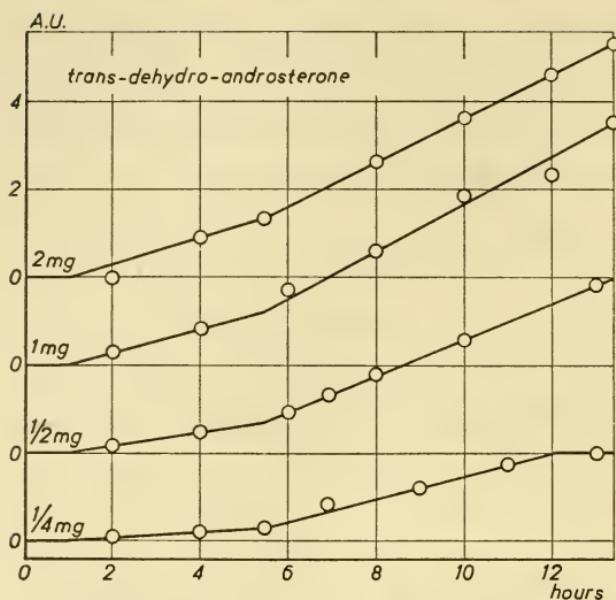


Fig. 22. Ovipositor growth curves after administration of *trans-dehydro-androsterone*.

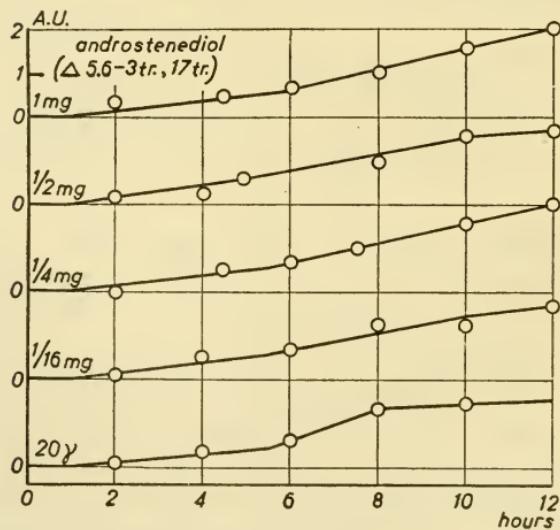


Fig. 23. Ovipositor growth curves after administration of androstenediol.

e. *Androstanedione-3,17*. With concentrations of  $1/16$ ,  $1/8$ ,  $1/4$ ,  $1/2$  and 1 mg per 750 ml water the following growth-curves were obtained (Fig. 24).

The characteristics of these curves are:—

Latent period, 1 hour. With higher concentrations, growth continues after 12 hours.

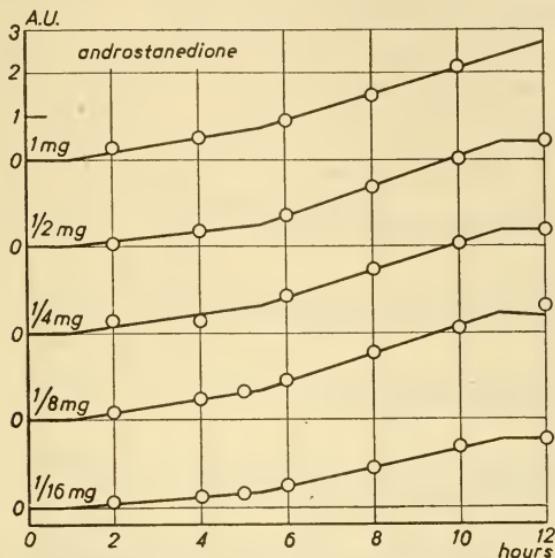


Fig. 24. Ovipositor growth curves after administration of androstanedione.

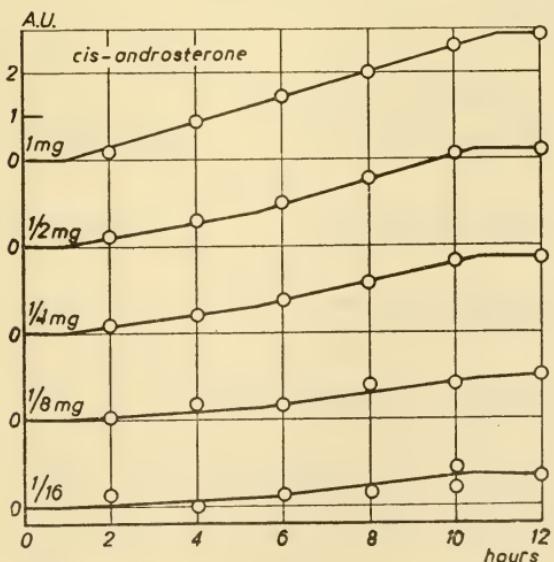


Fig. 25. Ovipositor growth curves after administration of cis-androsterone.

f. *Androstanol-3-cis-one-17* (*cis-androsterone*). With concentrations of  $1/16$ ,  $1/8$ ,  $1/4$ ,  $1/2$  and  $1$  mg per 750 ml water the following growth-curves were obtained (Fig. 25). The characteristics of these curves are:—

Latent period, 1 hour. Growth ends after 11 hours.

g. *Androstanol-3-trans-one-17* (*trans-androsterone*).

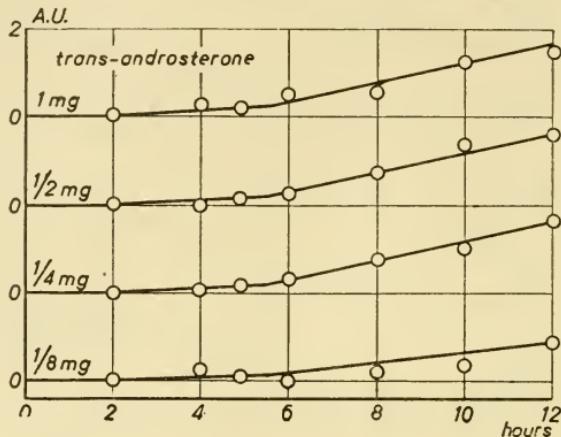


Fig. 26. Ovipositor growth curves after administration of trans-androsterone.

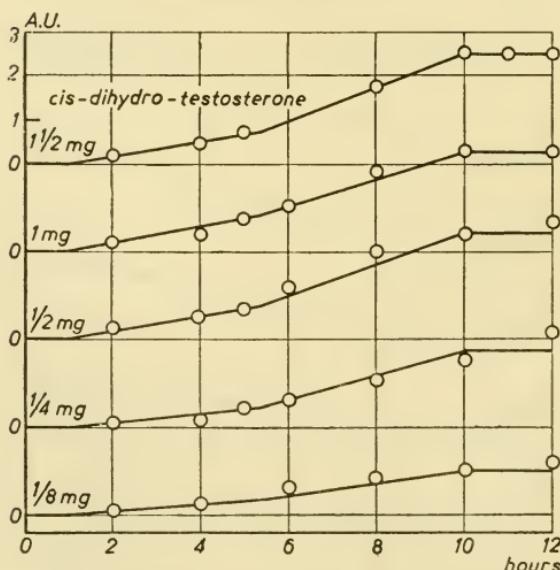


Fig. 27. Ovipositor growth curves after administration of cis-dihydro-testosterone.

With concentrations of  $\frac{1}{8}$ ,  $\frac{1}{4}$ ,  $\frac{1}{2}$  and 1 mg per 750 ml water the following growth-curves were obtained (Fig. 26). The characteristics of these curves are:—

Latent period, 1 hour. After 12 hours growth still continues.

h. *Androstanol-17-cis-one-3 (cis-dihydro-testosterone)*. With concentrations of  $\frac{1}{8}$ ,  $\frac{1}{4}$ ,  $\frac{1}{2}$  and 1 mg per 750 ml water the following growth-curves were obtained (Fig. 27). The characteristics of these curves are:—

Latent period, 1 hour. Growth ceases after 10 hours.

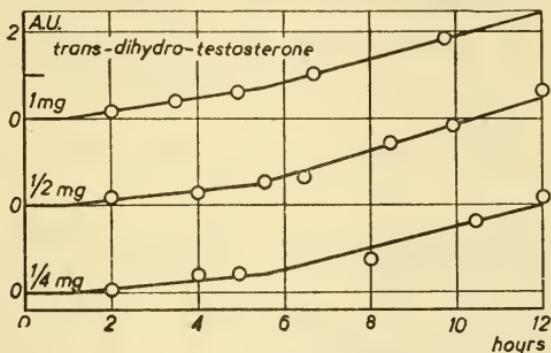


Fig. 28. Ovipositor growth curves after administration of trans-dihydro-testosterone.

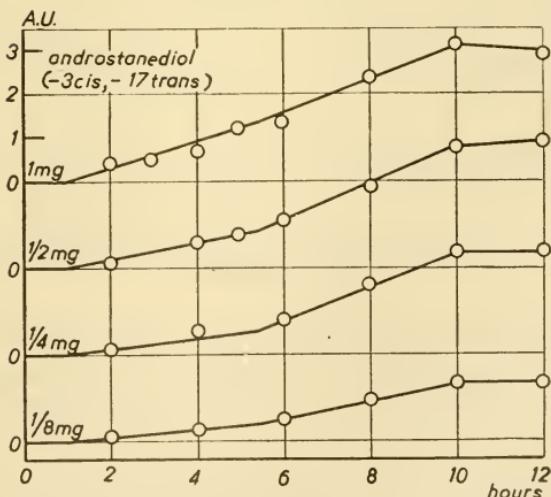


Fig. 29. Ovipositor growth curves after administration of androstanediol.

i. *Androstanol-17-trans-one-3* (*trans-dihydro-testosterone*). With concentrations of  $\frac{1}{4}$ ,  $\frac{1}{2}$  and 1 mg per 750 ml water the following growth-curves were obtained (Fig. 28). The characteristics of these curves are:—

Latent period, 1 hour. Growth continues after 12 hours.

j. *Androstane-3-cis, 17-trans-diol*. With concentrations of  $\frac{1}{8}$ ,  $\frac{1}{4}$ ,  $\frac{1}{2}$  and 1 mg per 750 ml water the following growth-curves were obtained (Fig. 29). The characteristics of these curves are:—

Latent period, 1 hour. Growth ceases after 10 hours.

k. *17-methyltestosterone*. With concentrations of  $\frac{1}{4}$ ,  $\frac{1}{2}$  and 1 mg per 750 ml water the following growth-curves were obtained (Fig. 30). The characteristics of these curves are:—

Latent period, 1 hour. Growth ceases after 10 hours.

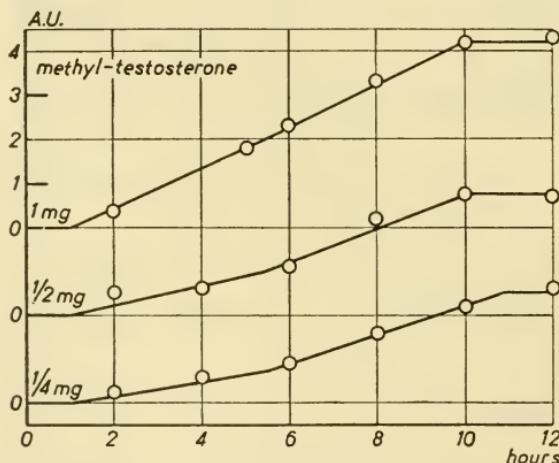


Fig. 30. Ovipositor growth curves after administration of methyl-testosterone.

l. *Testosterone-propionate*. With concentrations of  $\frac{1}{4}$ ,  $\frac{1}{2}$ , 1 and 2 mg per 750 ml water the following growth-curves were obtained (Fig. 31). The characteristics of these curves are:—

Latent period, 1 hour. This curve does not show the "crack" after  $5\frac{1}{2}$  hours, typical of male hormones. Growth ceases after  $5\frac{1}{2}$  hours.

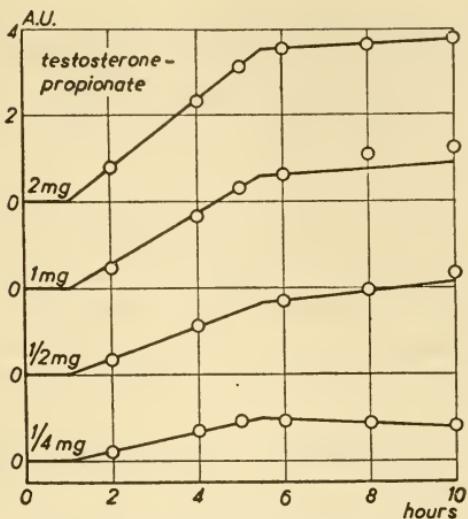


Fig. 31. Ovipositor growth curves after administration of testosterone-propionate.

On considering again the relation between the activity and the chemical constitution of the first 10 substances dealt with, we concluded that if one regards the two growing phases which, for androgenic substances, are expressed so

TABLE I

Androgenic substances	1st phase	2nd phase
4,5-androstenedione	3	1—3
4,5-androstenolone, 17-trans	6—8	1—3
5,6-androstenolone, 3-trans	1	1—3
5,6-androstenediol, 3-trans, 17-trans	2	4
androstanedione	4—5	8—9
androstanolone, 3-cis	6—8	8—9
androstanolone, 17-cis	4—5	5—6
androstanolone, 3-trans	10	10
androstanolone, 17-trans	9	7
androstanediol, 3-cis, 17-trans	6—8	5—6

typically as separate phases in the ovipositor test, the relation is less evident than in the case of the pregnanes. If we arrange the substances in order of decreasing activity, separating the 1st and 2nd growing phases, we get Table I (p. 25).

#### § 4. THE REACTION TO GLANDULAR EXTRACTS

In glands and in the fluids of the body, mixtures of hormones are nearly always present. If it is desired to use the ovipositor test to demonstrate the presence of a given "hormone" in a glandular extract, it should be borne in mind that the reaction of the ovipositor might be influenced by the various steroids present. Hence it is important to examine more closely the behaviour of such hormonal mixtures.

Experiments made in this connexion have shown that the effects of the separate components of hormonal mixtures are simply complementary. There is no question of antagonism between, e.g., androgenic and estrogenic substances. Evidently the hypophysis is capable of converting the stimulus received from each substance, resulting in the production of gonadotrophic hormone and growth of the ovipositor.

Since the estrogenic hormones all show a latent period of  $5\frac{1}{2}$  hours, and the androgenic steroids are effective only in very high doses, the ovipositor test is suited chiefly to the demonstration of steroids of the progesterone type, whose latent period is only 1 hour, the period of linear growth ending before the reactions caused by the estrogenic substances begin. It is also important to note that, of all pregnane derivatives, progesterone is the most active. *It is more than 1200 times as active as pregnanediol and all androgenic steroids.*

Of the organs examined, from which aqueous extracts were prepared and added to the aquarium water, the following proved to be inactive: cerebrum, duodenum, epiphysis, stomach, liver, posterior pituitary, spleen, pancreas, thymus, and thyroid. Positive results were obtained with suprarenal gland, corpus luteum, interstitial tissue of the ovary, placenta, and testis. Of the body-fluids, amniotic fluid reacted negatively. Positive reactions were obtained in most cases from blood, fluid from ovarian-cysts, follicular fluid, sperm, and

urine. Blood, cystic fluid and follicular fluid produced progesterone-like reactions. Urine produced typical ovipositor-growth-curves, which we attributed to the action of a substance we have called *luteidin* (*vide p. 35*).

a. *The reaction to testis-extracts.* We know that the testis contains testosterone, and also estrogenic substances. It might, therefore, be expected that the curve showing the growth of the ovipositor would be either of an androgenic or an estrogenic type, or a combination of both. The growth-curve obtained with an aqueous extract from the testis of a stallion (Fig. 32), shows quite a different growth-curve,

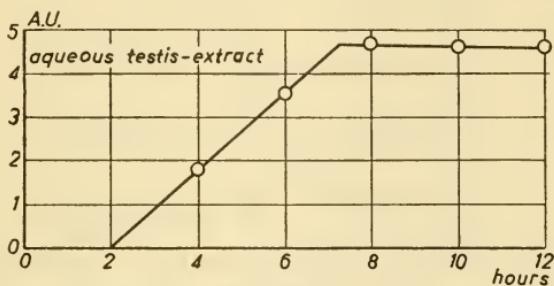


Fig. 32. Ovipositor growth curves after administration of aqueous testis-extract.

whose latent period is 2 hours. Since estrogenic substances all show a latent period of  $5\frac{1}{2}$  hours, the growth of the ovipositor cannot be explained in this way. The growth-curve further lacks the sharp bend at the  $5\frac{1}{2}$  hours' point, typical of natural androgenic hormones. Moreover, the growth manifesting itself  $5\frac{1}{2}$  hours after the administration of the extract is greater than could possibly be obtained by the combined activity of the androgenic and estrogenic hormones present in 10 g of testis tissue. The element active in the ovipositor test cannot, therefore, be either testosterone or estrogenic hormone. To judge from the shape of the growth-curve, a steroid from the pregnane group must play a part. The substance in question is either identical with, or similar to, 21-oxy-pregnanol-3-one-20 of HIRANO (1936). Guided by the ovipositor test, one might attempt to isolate this unknown substance.

*b. The reaction to extracts from the adrenal cortex.* As we know, a number of steroids have already been isolated from the adrenal cortex, without justifying the contention that the "true" cortex-hormone (if it exists as such) has been discovered. Fig. 33 shows the ovipositor growth-curve

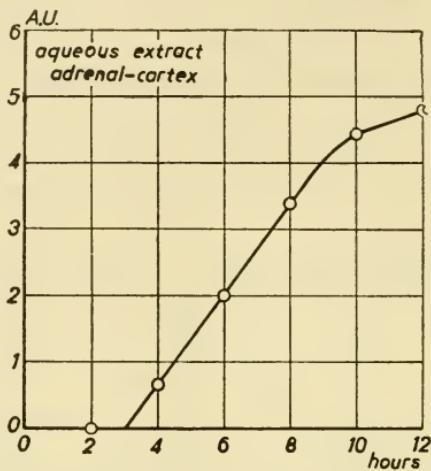


Fig. 33. Ovipositor growth curves after administration of aqueous adrenal cortex extract.

obtained with an aqueous extract from 4 g of adrenal gland tissue from a cow. The curve shows a latent period of 3 hours, while the period of linear growth continues until 9 hours after the start of the experiment. It is extremely improbable that the reaction is the result of corticosterone and/or progesterone, since in that case the latent period would have been 1 hour. It is also improbable that androgenic and/or estrogenic substances were involved, in view of the strong effect of only 4 g tissue-extract per 750 ml aquarium water. Even if these hormones were present in the water their action would be completely dominated by that of one or more steroids not identical with either progesterone or corticosterone—or desoxycorticosterone—or with the active component of Cortine "Organon", the growth-curves of which are shown in Fig. 34. The latter extract, however, produces curves with a latent period of 2 hours, while the period of linear growth is 5 hours. In Fig. 35, the typical differences

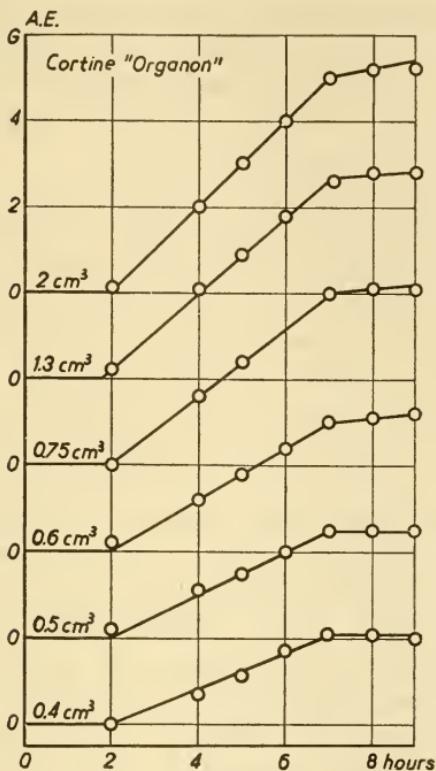


Fig. 34. Ovipositor growth curves after administration of Cortine "Organon".

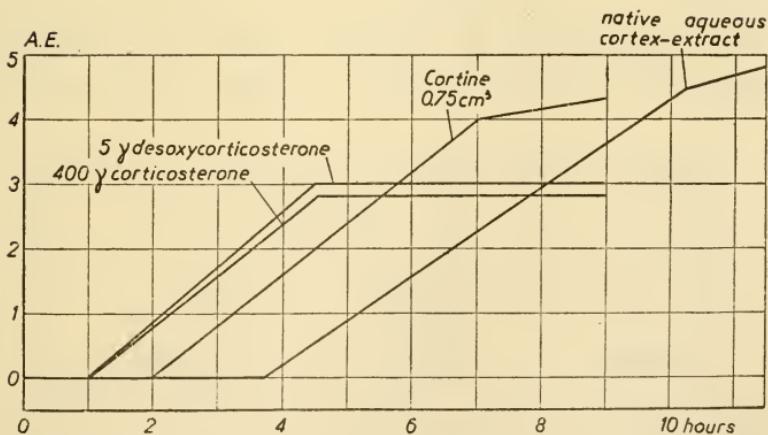


Fig. 35. Ovipositor growth curve after administration of synthetic adrenal cortex steroids and extracts. Note the marked difference between the respective curves.

between the growth-curves of Cortine "Organon", corticosterone, and desoxycorticosterone, as well as those from the aqueous cortex-extract are clearly shown. It might be possible to isolate the hormone of the adrenal cortex by means of the ovipositor test.

c. *The reaction to corpus luteum extracts.* With corpus luteum extracts growth-curves similar to those for progesterone were always obtained in the ovipositor test. When calculating the progesterone content per g of tissue, the averages of the values found appeared to correspond well to the values found, according to the literature, in the CLAUBERG and CORNER tests. Most probably, therefore, the activity of corpus luteum extracts in the ovipositor test is based upon the presence of progesterone. The other steroids are evidently present in too slight a concentration to interfere.

Since the ovipositor test is so sensitive to progesterone, we have tried to determine thereby the progesterone content of individual corpora lutea removed after ovariectomy in women. We had at our disposal 28 corpora lutea, whose probable age was determined on the basis of the cycle of the women concerned. Table II (p. 31) gives the values found. (For further particulars, *vide* DUYVENÉ DE WIT, 1942).

In Fig. 36, the progesterone contents found per gram of tissue, and related to the respective ages of the corpora lutea examined, are marked by means of crosses. The pyramidoid,

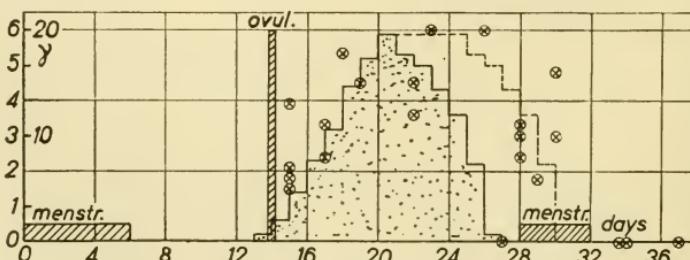


Fig. 36. Showing the connexion between (a) the progesterone-like hormone from a number of human corpora lutea, expressed in progesterone, and (b) the pregnanediol excretion according to VENNING and BROWNE, on corresponding days in the cycle.

punctuated figure represents the average secretion of pregnanediol by VENNING and BROWNE (1936) in 9 women. These values have been reduced, for each woman, to a

TABLE II

No. of the corpus luteum	Weight in grams	Age in days	Progesterone content of the whole gland	Progesterone content per gram tissue
1	2.2	1	16	7
2	2.1	1	10	5
3	1.25	1	8	6
4	2.1	1	28	13
5	0.65	3	5	8
6	2.2	3	24	11
7	1.1	4	20	18
8	2.6	5	38	15
9	0.67	8	8	12
10	1.6	8	24	15
11	0.9	9	18	20
12	1.5	12	30	20
13	2	13	0	0
14	1.3	14	13	10
15	1.4	14	16	11
16	1.8	14	14	8
17	0.85	15	5	6
18	2.4	16	24	10
19	1.3	16	21	16
20	0.1	20	0	0
21	0.7	20	0	0
22	0.45	23	0	0
23	0.05	24	0	0
24	0.6	24	3	5
25	0.2	25	0	0
26	0.25	28	0	0
27	0.3	52	0	0
28	0.15	53	0	0

standard cycle of 28 days. It is evident from this figure that the secretion of pregnanediol, representing the secretory activity of the corpus luteum, increases linearly from the time of ovulation until the seventh day of life of the corpus luteum. After this, secretion diminishes, gradually at first, then more quickly, ceasing entirely on the first day of the next menstruation.

With regard to the crosses representing the hormone content of the corpora lutea examined, it appears that they are placed in a zone whose form is about the same as that of the secretion-curve referred to above.

*During the first 7 days after ovulation, i.e., during the growth of the corpus luteum, it appears that there exists a distinct parallelism between secretory activity and progesterone-content of the corpora lutea. During the 7 days following, i.e., during the degeneration of the corpus luteum, it appears that the secretory activity diminishes faster than the hormone content.*

This phenomenon no doubt results from the fact that, during the decreased production of hormone, the corpora lutea still retain their normal stock of hormone for some time, until degeneration of the tissue has proceeded far enough for the hormone to disappear. One has the impression that the decrease in hormone content sets in about 4 days later than the decrease in hormone production. (Vide the dotted line in Fig. 36, shifted 4 days to the right).

The hormone contents found suggest that further investigations should be made into the question of whether the substance present in the corpora lutea and active in the ovipositor test should be attributed either wholly or partly to progesterone. In the latter case the reactions found should be the sum of the reaction to progesterone and those to the remaining active agents. The ovipositor test should then, generally speaking yield higher values than is consistent with the amount of progesterone present in corpora lutea. In the ovipositor test, reactions are obtained, per gram of tissue of mature human corpora lutea, which correspond to those of 10—20  $\gamma$  progesterone. CLAUBERG (1932), in his experi-

ments on rabbits, obtained barely positive reactions with an extract of 55 g human corpora lutea, which amounts to a progesterone content of 12—16 γ per gram. The values found with both test-methods, therefore, are of the same order of magnitude, so that the substance measured by means of the ovipositor test is most probably progesterone.

There is a potential source of error, in testing corpus luteum extracts, arising from the possible adsorption of progesterone by drops of fat dispersed in the water. If, in our experiments, this should have occurred to an extent upsetting calculation, the result would, on the one hand, show a too low progesterone content; this, however, would be compensated by additive substances whose growth curve shows a great similarity to that of progesterone.

*d. The reaction to extracts from the ovary.* Since the interstitial gland, according to ASCHNER, is most strongly developed in the most primitive animals and still largely replaces the corpus luteum functionally, one may expect good ovipositor reactions with extracts from the ovary of the lower mammals. Granting further that ontogeny here constitutes a repetition of phylogeny, it should also be possible to obtain ovipositor reactions from premature ovaries.

The four ovaries from a pair of still-born twins from the 6th month of pregnancy, and (separately therefrom) the 2 uteri, were extracted. The ovaries, which together weighed 0.4 g, caused in 6 fishes an average ovipositor growth corresponding to that after administration of 4 γ progesterone, i.e. 10 γ per gram of tissue (*vide* Fig. 37). The extract from the uteri produced no reaction.

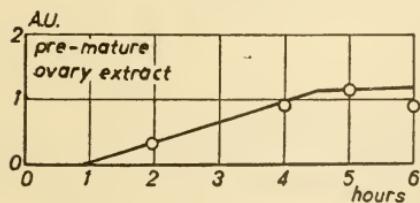


Fig. 37. Growth-curve obtained with the lipoid extract from two pre-natal human ovaries.

The 2 ovaries of a full-term still-born child were also examined. The ovaries, which together weighed 0.5 g, caused an average ovipositor growth corresponding to that of 20 γ progesterone, i.e., 40 γ per g of tissue.

The ovipositor reaction obtained with the older fœtus, therefore, was stronger than that with mature human corpora lutea! This discovery is of some significance relatively to CLAUBERG'S view (1937), according to which the secretions of primordial follicles should be held responsible for the blood-supply and growth of the developing infantile uterus. Whether or not the hormone involved is identical with progesterone might be made the subject of further investigation. Such investigation would be of importance in ascertaining whether there exists a special "interstitium-hormone".

### § 5. THE REACTION TO URINE

When the urine of pregnant women is added to the aquarium water, the ovipositor growth-curve shows a totally different character from that obtained after the administration of the steroids already referred to. Thus, with concentrations of  $\frac{1}{4}$ ,  $\frac{1}{2}$ , 1, 2, 3 and 5 ml urine per 750 ml water at  $22^{\circ}\text{C}$ , the following growth-curves were obtained (Fig. 38). The characteristics of these curves are:—

Latent period,  $5\frac{1}{2}$  hours. Growth ceases after 12 hours.

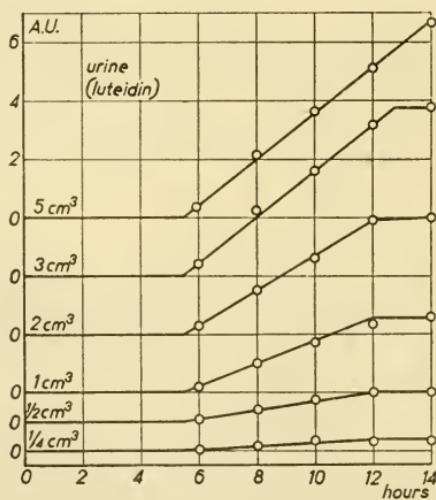


Fig. 38. Ovipositor growth-curve obtained with the urine of a non-pregnant woman. Latent time:  $5\frac{1}{2}$  hours.

If one compares the curves obtained with the urine, with those obtained with progesterone, androsterone and estrone, then the result is as shown in Fig. 39. Since, out of 27 steroids

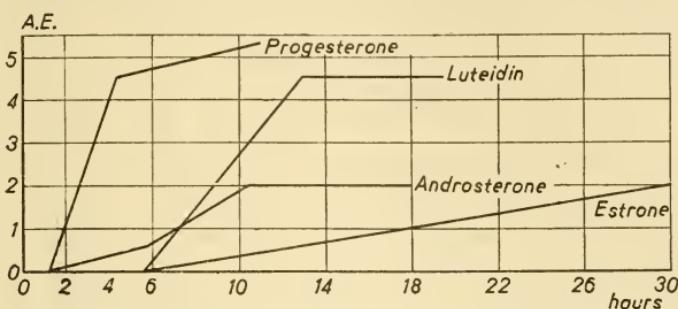


Fig. 39. Ovipositor growth-curve obtained with relatively large quantities of progesterone, androsterone, luteidin and estrone. Note the typical differences in latent time and duration of linear growth.

examined, none gives a growth-curve resembling those of urine, we are compelled to assume that the ovipositor test has proved the existence of a new hormone or hormonal derivative, which we have provisionally called luteidin (BRETSCHNEIDER and DUYVENÉ DE WIT, 1937).

*Luteidin secretion in women.* Luteidin is secreted both by men and by women; in men however, less than in women. Secretion is not conditional upon pregnancy, for during the menstrual cycle, luteidin is produced. In Fig. 40

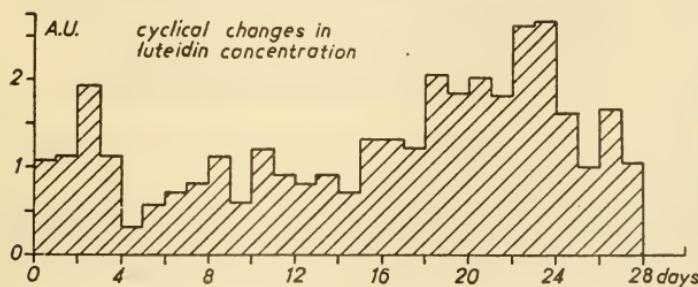


Fig. 40. Average luteidin concentration per 3 ml urine of a number of normal, non-pregnant women during a cycle converted to a 28 days' standard. The maximum falls within the corpus luteum phase.



we give the average luteidin concentration per 3 ml urine in 750 ml water (expressed in A.U.) of a number of non-pregnant women. In this, the cycles have been reduced to one of 28 days. It appears that there is a decrease in luteidin concentration towards the end of menstruation. From the 6th until the 10th day there is a slight rise. At about the time of ovulation the degree of concentration remains constant. From the 16th to the 25th day the concentration increases almost continuously until a maximum is reached. From the 25th to the 28th day it falls again. On comparing this secretion-curve with that of the gonadotrophic hormone (ZONDEK, 1931), and that of the follicular hormone (SMITH c.s., 1938; SIEBKE, 1930; FRANK c.s., PEDERSEN-BJERGAARD, 1936; PALMER, 1937-'8), it will be seen that it is characteristic. The same applies to that of the secretion-curve obtained with pregnanediol (VENNING c.s., 1936) and male hormone (LAQUEUR, 1936).

According to KANTER, BAUER, and KLAWANS (1934), the ovipositor test might be used for pregnancy diagnosis. In order to verify this we examined the urine of 112 pregnant women. The luteidin concentration in 3 ml of urine per 750 ml water, taking an average of all the months of pregnancy, is shown in Fig. 41. In the second month of

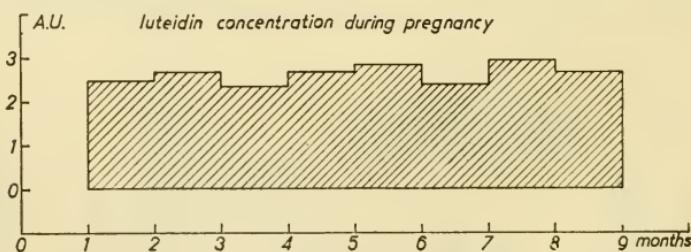


Fig. 41. Average monthly luteidin concentration per 3 ml urine of a number of pregnant women, showing the degree of concentration to remain practically constant all through pregnancy.

pregnancy it does not differ much from that in the last month. It therefore remains almost constant during the entire

period of pregnancy. Luteidin thus differs clearly from pregnanediol and estrone, the secretion of which increases considerably during pregnancy. During pregnancy luteidin is no longer secreted cyclically, as is apparent from Fig. 42.

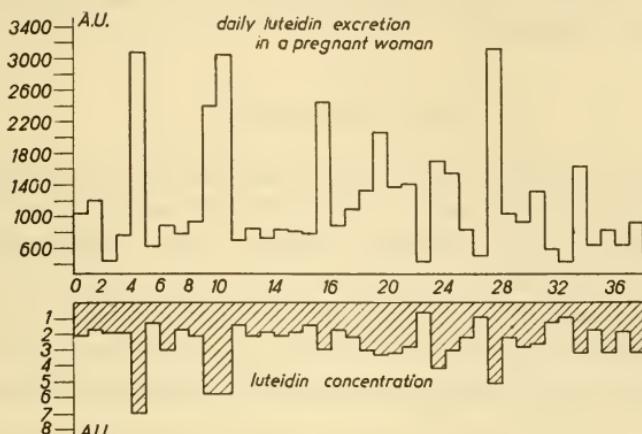


Fig. 42. Daily luteidin excretion in a normal pregnant woman during 6 weeks. Against this, the luteidin concentration per 3 ml urine. No periodicity observable in the excretion.

Here the daily urine of a pregnant woman was examined.

The luteidin secretion in the normal cycle during pregnancy and lying-in is given again in Fig. 43. We do not know of any sexual hormone from which a curve of this kind could have been obtained. This supports our assumption

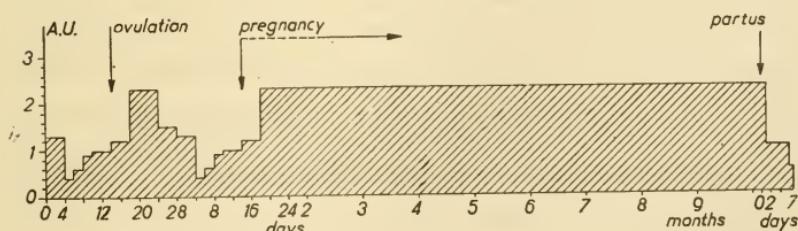


Fig. 43. Schematic representation of the luteidin concentration in 3 ml urine during a period of 28 days; half a period (14 days); subsequent pregnancy, and lying-in.

that we are dealing with a new hormone or hormonal derivative.

As the concentration of luteidin during pregnancy is equal to that during the corpus luteum phase in the absence of pregnancy, it is no criterion for the determination of pregnancy. For this reason the ovipositor test is not suitable for pregnancy diagnosis.

#### § 6. SUMMARY

We have concluded that, given certain conditions and precautionary measures, the ovipositor test can be used with advantage for certain endocrinological assays. Although more than one mammalian hormone may react positively in a particular test, it may be possible to ascertain from the form of the growth- and/or concentration-curve which is the active hormone. Its advantages are the speed of the reaction (usually within 12 hours), and the fact that the fishes may be used several times in succession.

The ovipositor test is, in our view, especially suited to the demonstration of the presence of small quantities of progesterone, e.g., in single human corpora lutea. It may also be used to demonstrate the presence of substances not identical with either testosterone or corticosterone, or their derivatives, in the tissue of the testis and the adrenal cortex.

By means of the ovipositor test the presence of a substance not corresponding to one of the known steroids, and to which we have given the name of luteidin may be demonstrated in human urine. Luteidin is a hydrolysable substance secreted cyclically in non-pregnant women, and in maximum quantity during the corpus luteum phase. During the whole period of pregnancy the secretion of luteidin remains constant, and shows the same level as that during the corpus luteum phase. For this reason the ovipositor test is not suitable for pregnancy-diagnosis.

The ovipositor test is, however, of particular significance for the examination of the sexual hormones of lower vertebrates, which are different from mammalian hormones.

We doubt whether it is possible to produce reactions in mammals by the administration of sexual hormones produced by fishes (oviductin and copulin, (*vide* p. 56 and 131). In fishes, however, the ovipositor test is *adequate*, and may therefore be regarded provisionally as *an appropriate method for both qualitative and quantitative hormonal analyses in non-mammalian vertebrates*.

### § 7. THE LUTEIDIN PROBLEM

*Luteidin* is the name given by DUYVENÉ DE WIT to a hitherto unknown component of urine, both of men and women, which as yet can be identified only by a peculiar growth-curve in the ovipositor test, which growth-curve cannot be produced by any of the known active hormones generally present in urine, either separately or unitedly, the form of this growth-curve being determined by the following data:—

- a. the latent period is  $5\frac{1}{2}$  hours;
- b. after this, very marked and rapid growth sets in,
- c. which growth reaches its maximum after about 12 hours, and may — all according to the sensitivity of the fishes — amount to as much as 7 A.U. per 3 ml of urine  $22^{\circ}$  C.

Maximal growth may even be obtained sometimes with the addition of 1 ml urine to 750 ml aquarium water.

In the course of a systematic investigation, DUYVENÉ DE WIT has shown that a growth-curve of this sort differs essentially from that produced by 27 different steroids, amongst which were estrogenic and androgenic substances: progesterone, pregnanediol and cortical-steroids. In the case of pregnanes and androstanes, the latent period is much shorter, whilst in the case of estrogenic steroids — again the only group of substances with the same latency time of  $5\frac{1}{2}$  hours — the maximum of growth is lower and much more protracted. The united participation in the total growth, on the part of steroids normally present in urine is only small; in

the case of urine rich in steroids — e.g. towards the end of pregnancy — it would be in the neighbourhood of 1 A.U., as measured 12 hours after the urine is added to the aquarium water.

On the basis of these facts DUYVENÉ DE WIT assumed that there is present in urine an unknown, strongly active element, characterized only by its action in the ovipositor test. He measured the concentration of luteidin of a large number of samples of urine, expressed in A.U. per ml of urine, and arrived at the conclusion that the secretion, in women, is abundant and fairly constant during pregnancy, and at other times variable, with a maximum during the corpus luteum stage, when pregnancy values are reached.

Now what kind of substance does this word "luteidin" denote?

To supply the answer to this question, HEINTZBERGER made investigations, which, however,—owing to various causes—could not be terminated.

Luteidin is a substance soluble in water and, in this solution, labile, as is evident, for example, from the fact that active urine, with a latent period of  $5\frac{1}{2}$  hours, when kept at room temperature for 2 days, still produces the same growth as before, but *that the latent period is then reduced to 2 hours*. The entire curve is shifted forward without any change in its form, so that a single measurement, e.g. after 12 hours, does not even betray this fact. This shortening of the latent period may also be obtained by just bringing the urine to the boil. In any attempt at isolating luteidin, therefore, it is certain that none of the usual methods of urine extraction can be applied which consist in the acidification of the urine with concentrated HCl, followed by prolonged extraction with ether or benzene on the steam-bath. When kept in a refrigerator, however, the urine remains unchanged.

Testing is done at a temperature of  $22^{\circ}$  C, i.e., slightly higher than room temperature. This gave VAN KOERSVELD, who performed the tests, the idea that it might be possible to measure the disintegration-rate of luteidin. To this end he added active urine to the aquarium water, but did not put

in the fishes until some time afterwards, so that any hydrolysis of the active component of the urine might proceed unhindered before the actual test began. In this experiment he actually found a reduction of the latent period, usually a little less than the time during which the urine had been at 22° C, before the fishes had entered the water. Finally, a constant latent period of 2 hours was reached.

Attempts at extraction of luteidin at room temperature with ether or benzene were without result, because these extracts were invariably inactive; it was found, however, that an extract could be made with butanol, in the cold, which contained all active substances from the urine. To this end the urine is shaken up with butanol three times; the resultant emulsion is disintegrated by centrifugation; the solvent is evaporated in a vacuum, and the residue is put in alcohol for testing purposes. The urine extracted is invariably completely inactive. The latent period of the active extracts is always 5½ hours, and retains this value until the extract is dissolved in water, for then hydrolysis sets in again with its measurable rate.

All this seems to point to the fact that luteidin itself may be an inactive substance, the active component being freed only by hydrolysis. This is not surprising, since it is well known that substances strongly active physiologically are often inactivated by the body through coupling to another substance before being secreted into the urine. Thus, estrone is secreted in the form of inactive estrone-sulphate, and estriol in the form of glucuronide.

The identity of the active component of luteidin, however, has not yet been determined. Actually, of all the steroids examined, only allo-pregnadiol, 3-20 produces a curve resembling in any way that of hydrolysed luteidin: a latency period of 2 hours, linear growth for 5½ hours; the maximum of growth, however, is only about 3.2 A.U., and is attained only with quantities above 225 γ.

Well then: a urine, 2 ml of which already produces maximal growth would have to contain 500 times this quantity, or 110 mg allo-pregnadiol per litre, whereas the

highest value ever reached by the end of pregnancy is only about 15—29 mg/l. For that matter, it already follows from what has been said with respect to the secretion in both men and women that we could hardly have to do here with this substance.

What did, however, become clear from this investigation was that the active portion of the luteidin molecule finally arrives, during the purification of the urine-extracts, in about equal quantities, in the neutral fraction which also contains the androgenic substances, and in the weakly-acid (phenol) fraction, in which the estrogenic steroids are also contained.

Indeed, everything points to the presence in urine of a not yet identified substance with a high activity in the ovipositor test, although it may, of course, be possible that the substance in question is known as such, but that its biological activity has not yet been revealed.

The facts described above having been realized, we proceeded to extract 100 l urine of pregnant women, with a view to using this extract as initial material in an attempt to isolate luteidin. It is true, that, in this urine, there are large quantities of estrogenic steroids, pregnanediol and kindred substances; an advantage, however, is that luteidin secretion is both high and constant, so that there is no risk of obtaining large quantities of urine extract which subsequently turn out to be nearly or wholly inactive.

The first experiments were made with a crude extract of 25 l urine, whilst at the same time the remaining 75 l were extracted. As is usual in such experiments the comparative solubility in the various solvents was first examined. There is not, however, very much choice, as it appears that shaking up the butanol-extract with ether, benzene and suchlike eliminates practically no impurities (although any *free* estriol, etc. is removed); the "dry-rest", therefore, hardly diminishes at all. After a few trials, however, it was found that a purification of roundabout five times is to be obtained by shaking out the butanolic solution of the extract with 0.1 N KOH. The luteidin then remains in the butanol-phase. Into the alkaline fraction go, e.g., the estrogens, which should be

present in 2 ml urine in a quantity of about  $10\gamma$ . This quite explains the low activity of this fraction.

After an alcoholic solution had been made of the neutral butanol fraction, we found that, when this had been left untouched for a week-end at a fairly low temperature, crystals had separated from it, which could be burned without leaving any residue, began to frizzle when heated to  $250^\circ\text{C}$ , and melted, whilst disintegrating, at  $255-260^\circ\text{C}$ . After back-crystallisation the melting point was  $264^\circ\text{C}$ . These properties point in the direction of Na-pregnane-diol-glucuronide, which, in the pure state, melts at  $268-271^\circ\text{C}$ . No significant biological activity was expected of this substance; we therefore first tested the solutions left over. The result was that both the neutral and the acid fraction as well as the layer of water remaining during the operation were *inactive*! This result surprised us not a little, for evidently the active element had disappeared from the extract, so active during the initial experiments. The only possibility, to our minds, was that the isolated pregnane-complex, against all expectations, should prove to be active all the same. And indeed, during a provisional test with 3 mg, a growth of 5.5 A.U. was observed, with a latent period of  $5\frac{1}{2}$  hours. When fresh fishes were put in the same water a few days afterwards, the growth was, if possible, a little greater; the latent period, however, had gone back again to 2 hours. The quantity of material used corresponds to 2 mg pregnane-diol, which, according to DUYVENÉ DE WIT, should produce a growth of 3 A.U., but only after 10 hours, including a latent period of 1 hour.

We are therefore faced with the question: what is the reason why this pregnane-diol-glucuronide which we have isolated is so much more active than pregnane-diol itself, with which we are familiar? It was evident from a few experiments that glucuronic acid is completely inactive in the ovipositor test, and cannot, therefore, be held accountable for the marked increase in activity on the part of pregnane-diol. The only way to find the answer to the above question is, therefore, by isolating the pregnane-complex in sufficient quantity and examining it as to its properties, after adequate

purification and under control of elementary analysis.

Pregnanediol glucuronide was precipitated, by means of acetone, from the extract purified by shaking out with alkaline, according to the method of VENNING; as a matter of fact we had already found out during these investigations that acetone precipitates the luteidin. In this way a brown-coloured crystalline substance was obtained from the 75 l urine, in a quantity of 67 mg/l, a value which is normal for the urine of a pregnant woman. The melting point was about 220° C, and the substance disintegrated. Purification did not go smoothly; only with much difficulty did we succeed in obtaining a few fractions of low weight, and coloured almost white. These crystals did actually contain glucuronic acid, as was proved by the colour-reaction of TOLLENS. With naphthoresorcinol and concentrated HCl, a bluish-violet colour appeared, which could be shaken out with ether. The colour reaction, however, turned out to be unsuited to quantitative determinations, so that the presence of any foreign substance could not be ascertained in this way. For, the curious thing is that these thoroughly purified crystalline fractions, too, are still highly active; here, moreover, we can observe the characteristic property of luteidin, i.e., reduction of the latent period, although, as is known, the necessary condition for the hydrolysis of pregnanediol is that it is acidified and boiled.

The crystals obtained from the 25 l extract were darker in colour than those from the 75 l extract, and yet they had a higher melting point. The melting points were 260—263° C and 240—246° C, respectively.

Part of the, still impure, fraction was hydrolysed by boiling with HCl; from this white crystals were obtained, with a melting point of 222° C, after it had been noticed that, at 180° C, the material began to sublimate, and redeposit itself in another form of crystal. In the case of pregnanediol, 225° C is usually found, whilst the value for the pure substance is 235° C. When, however, 16 mg of the substance obtained was acetylated with acetic acid anhydride and some pyridine, an acetate was obtained with a sharp melting point of 161.5—162° C; a value lying exactly between that of the ordinary,

and that of the allo-pregnane diol-acetate; the values given for these being, respectively, 182° C and 140° C.

We may finally mention that the two crystalline fractions did not prove to be quite ash-free (this is, indeed, a well-known point of difficulty with pregnanediol-glucuronide), so that the elementary analysis could not give us any precise figures with regard to the constitution of the crystals. It was remarkable, however, that, the H % was about 4 % too low, as calculated relatively to Na-pregnane diol-glucuronide, the C % lay up to as much as 19 % below the value expected, which cannot be explained by the mere admixing of NaCl and Na<sub>2</sub>SO<sub>4</sub>.

Rather does the conclusion seem to force itself upon us that there must be a second substance present in the pregnanediol complex, which cannot be removed by recrystallisation, and present in different quantities in the two crystalline fractions. This would explain not only the difference in the melting points, but also the fact that the highest of the two melting points is still 8° too low.

A concentration-curve was determined of crystals melting at high temperature. The sharp bend lay at a quantity of 380 γ, which means that, already with this quantity, per 750 ml aquarium water, the maximal growth of 5, and on one occasion even 7 A.U. was obtained, whilst DUYVENÉ DE WIT, discussing the results of the testing of pregnanediol, remarks that only with the high concentration of 2000 γ per 750 ml water could a distinct reaction be obtained. Further investigation will, therefore, be necessary for the solution of the luteidin problem.<sup>1</sup>

<sup>1</sup> In the *Bioch. J.*, 40, (1946), 53, MARRIAN and GOUGH stated that they had isolated pregnane 3 (α)-ol-20-one from the products of hydrolysis of "sodium pregnanediol glucuronide", a water-soluble derivative which was persistently present in the latter as a 20 % impurity, and could not be removed by further 'purification'. This impurity may perhaps be identical with our "luteidin".

## II. THE CONCATENATION HYPOPHYSIS→OVARY →OVIPOSITOR IN THE BITTERLING

### § 1. THE HISTO-PHYSIOLOGICAL EXAMINATION OF THE HYPOPHYSIS

As we observed, it is possible to induce growth of the ovipositor in the female bitterling by the administration of estrogenic (mammalian) hormones. Further investigation showed that the reaction of the ovipositor takes place not only after administration of estrone, but also after administration of progesterone, testosterone and desoxycorticosterone; in short, all *estranes, pregnanes, and androstanes*.

As injection of an aqueous extract of the hypophysis of the carp caused marked formation of corpora lutea as well as growth of the ovipositor in the female bitterling, we suspected that this growth might be regulated by the hypophysis, through the corpora lutea. The latter will be dealt with in greater detail later. The possible rôle of the hypophysis was investigated (1) by a histological and cytological study of the hypophysis and by observation of the changes occurring simultaneously in the ovary and in the ovipositor, and (2) by noting the effect of hypophysectomy.<sup>1</sup>

<sup>1</sup>VAN OORDT and BRETSCHNEIDER investigated the possibility of developing premature sexual maturity in sexually immature eels (*Anguillæ* from 30-37.5 cm long, abt. 5½—8 years old) by administering gonadotrophes. To this end they used Ambinon "Organon", which consists of an extract of the anterior mammalian pituitary, in combination with Pregnyl "Organon", i.e., an extract from the urine of pregnant women and the pituitary sap of sexually mature, spawning carps, the hypophyses weighing together 1½ g. It was shown that the infantile testis of the eel is thereby stimulated to form spermids after the administration of an extract of 2-6 carps' pituitaries, and also by 2000 I.U. Pregnyl combined with 400 I.U. Ambion. Only an unphysiologically high dose of mammalian hormone, therefore, can have effect on the eel. This proves once again that, in investigating the endocrinology of non-mammalian vertebrates, it is preferable to use hormones which are specific to the species investigated.

*a. Cellular changes in the gonadotrophic zone.*

For reasons which will be explained in detail in § 2, we divided the hypophysis of the bitterling into *lobus tuberalis*, *anterior*, *intermedius* and *posterior*. The so-called "gonadotrophic zone" of the *pars anterior* was examined after differentiation by azan staining, which turns acidophil cells red, basophil cells blue, and chromophobe cells a pale-bluish tint. The staining capacity of the pituitary cells is rightly held to be related to the cellular function.

On examining the hypophysis of a bitterling caught during the winter months we noticed that its anterior lobe consisted almost entirely of acidophil cells. Only a small number of cells were of the basidophil or the chromophobe type. The number of basophil cells, therefore, was far too small to play an important part. The picture presented by the hypophysis changed in a surprising way, however, when we examined the fishes during their reaction to steroid hormones. Then, in certain parts of the anterior lobe, large "islands" of cells became suddenly, and pronouncedly, basophilic, whereas, in the control-animal, only acidophil cells were found in the same region (Fig. 44A). In addition to this, an estimate

Fig. 44A. Medial section through hypophysis of *Rhodeus amarus*. The dark parts are composed of basophile cells.



had already shown that, within a test period of 44 hours, the numbers of newly-formed basophil cells was subject to considerable fluctuation, but the place where they occurred was always the same.

In all these experimental animals the *pars anterior* was clearly divided, as regards colour, into two halves, one lying obliquely behind the other. The anterior half remained acidophilic; the posterior half, though not always sharply outlined against it, was distinctly separate, at the back, from the *lobus intermedius*, and contained basophil cells. It is exclusively in this zone, running obliquely through the hypophysis, that the changes occur which, either directly or indirectly, are caused by the exogenous hormone stimulus, and which synchronise with the changes in the ovary and with the reaction of the ovipositor. There is periodic alternation of acidophil and basophil cells. For this reason we felt justified in calling the region in question the "gonadotrophic zone".

Two observations support this view. In the first place the male bitterling has a considerably smaller anterior lobe than the female. In the male, too, the gonadotrophic region is far less developed. The sexual organization of the male does not require such an extensive gonadotrophic zone; testicular activity covers a much longer period (autumn and winter) and the stimulus is, therefore, much weaker per unit of time than in the female, which, during the spawning season, has to put the whole of its sex-apparatus into action quickly.

A second point in favour of our view is based upon the communication by MATTHEW (1936), who described the seasonal changes of the hypophysis in *Fundulus*, and observed that from September to December the cells of the "Uebergangsteil" (by which is meant the gonadotrophic region; *vide* p. 47) are acidophilic, whereas during spawning time (from May to July) they are chiefly chromophobe cells. When we reflect that the chromophobe phase represents part of the restitution phase of the pituitary cells, it is evident from his communication that this part of the hypophysis plays its part in the phenomena of the spawning period.

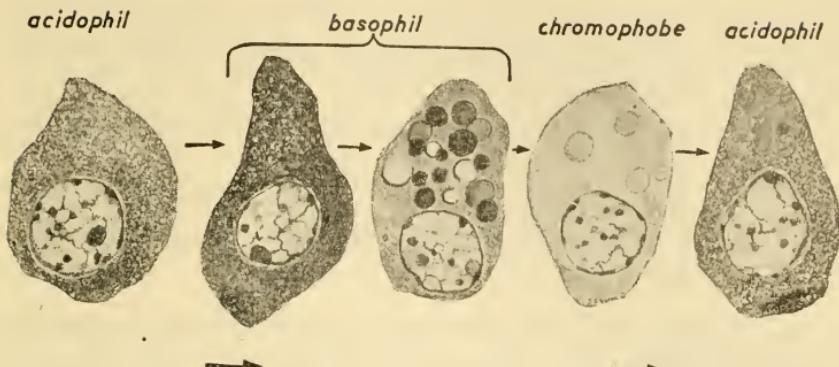


Fig. 44B. Showing the change of acidophile cells of the gonadotrophic pituitary zone into basophile, and back again into acidophile cells.

We shall now examine more closely the *cellular changes in the gonadotrophic region*.

The resting-stage of the gonadotrophic cells of the anterior lobe is characterized, in the control-animal, by acidophilia (Fig. 44B). Shortly after the first exogenous stimulus the cells suddenly turn markedly *basophilic*. After some time, however, they revert to *acidophilia*. It would seem from this that the *intermediate basophil stage* is probably the actual working phase of the cell and represents the restitution phase of the product, i.e., the luteinisation hormone. Without further study of the finer cytological changes we may, on the basis of colour differentiation, present the following provisional survey of the process:—

1. *Accumulation stage.* The cells are homogeneously filled with an acidophil secretion.
2. *Extrusion stage.* Inter-cellular acidophil secretions occur in the form of drops.
3. *Restitution stage.*
  - a. The emptied cell remains about the same size, and owing to its very slight affinity for stains, is *chromophobe*;
  - b. The cells are diffuse, but coloured distinctly *basophil*;

- c. Ring-shaped formations occur in their plasma, resembling GOLGI-systems, together with granulosa coloured a deep blue;
- d. In addition to the decreasing number of granules, numerous light vacuoles occur;
- e. Basophilia grows weaker and gradually gives place to acidophilia. Side by side with basophil granules, and vacuoles occur in the plasm.

4. *Transition.* The cell finally becomes homogeneously acidophil again. The rings and granules arising during the working phase might therefore be regarded as a product preceding the luteinisation hormone, the acidophil granules and vacuoles, and, finally, the diffuse, extruded drops constituting the actual incration. *Cellular activity fluctuates between these two working phases* since the first secretion of hormones is followed by a restitution, and when this is finished and the organ is stimulated afresh, another yield ensues; in other words *a rhythm is created*.

These rhythmic fluctuations were determined as follows. The hypophyseal sections were projected on to a sheet of paper  $35 \times 25$  cm. The basophil cells were traced (Fig. 45). The islands were blackened with marking ink, and the blackened area measured in relation to the remaining quantity of white.

We used an instrument which indicates, by means of two thermal elements and a galvanometer, under a constant amount of light, the degree of blackening as a galvanometer reading. In order to obviate errors arising through possible fluctuation in the intensity of the light, a calibrated scale



Fig. 45. Basophile cell-islands in the gonadotrophic zone; respectively 0, 15, 30 mins. and 2, 8, 10 hours after administration of progesterone.

of standard shades was made, by blacking on similar paper a progressive sequence of  $2\frac{1}{2}$ , 5, 10, etc.  $\text{cm}^2$  with marking ink. As control, a standard figure corresponding to the hypophysis picture was selected after each measurement. In this way the values of the different series could be compared with each other. During a pre-examination it was shown that a medium and a lateral section sufficed for quantitative determination. We took more than two sections, however, and calculated the average value therefrom. We thus obtained a definite value for each hypophysis, for the total number of basophil cells, on the basis of which a curve was constructed.

We shall now consider what this curve expresses. We should have preferred to determine the moment and the quantity of hormone extrusion; but this is impossible both for the ovary and the hypophysis. What can be measured, however, is the restitution of a quantity of hormones previously secreted. Thereby a loss of substance from the pituitary cells is registered, after the event, and at the same time the preparation for the next extrusion. Since this restitution phase lasts for some time, it may be assumed that the hypophysis curve derived from it "lags" slightly behind the curve representing happenings in the ovary (to be discussed later), in spite of the fact that the hypophysis is responsible for the changes in the ovary. Neither should we lose sight of the fact that, although the hypophysis, by secreting hormones, may react immediately to an exogenous stimulus, it is possible that the conditions necessary for a reaction may not exist in the ovary; for instance, that there are not sufficient large—i.e., luteinisable—ova present.

The hypophysis curves may be drawn in two ways:—  
(a) we may regard the anatomically limited gonadotrophic region, in which nothing else is happening, as a closed unit, in which *two kinds of cells are seen*, i.e., the *acidophil cells*, which still contain hormone and may secrete it, and the *basophil cells*, which are in the restitution phase, and do not contain hormone. The proportion between the number of cells in these functional periods is expressed in a curve in which the hormone content (acidophilia) is plotted against the loss (basophilia). The curves obtained in this way represent the relation between pituitary activity and the formation of

corpora lutea. If (b), on the other hand, one draws a reciprocal curve, by determining the increase in basophil cells, then one is able to compare it with the curve for corpus luteum formation, and observe synchronisation and rhythm.

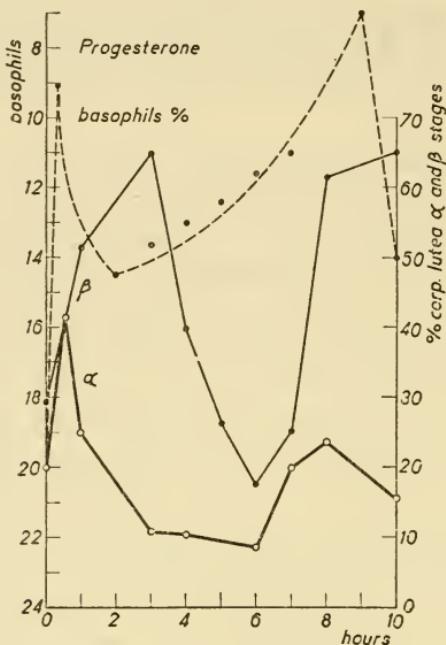


Fig. 46. Ratio between the quantity of basophile pituitary cells and corpora lutea in the  $\alpha$  and  $\beta$  stages after administration of progesterone.

Fig. 46 shows the curves obtained from an experiment with progesterone. The lower curve shows the growth of the ovipositor, as measured in A.U. (for the definition, *vide* p. 5). The  $\bigcirc$ — $\bigcirc$  curve shows the percentage of corpora lutea passing through the  $\alpha$ -phase (*vide* p. 82), and the  $\bullet$ — $\bullet$  curve, the percentage of those in the  $\beta$ -phase. The  $\bullet$ — $\bullet$  curve represents basophil cells. 15 minutes after the administration of progesterone the number of basophil cells reached a maximum. Within these 15 minutes, therefore, the first extrusion took place. Restitution followed within 2 hours. In 2 to 9 hours a second extrusion took place, the maximum number of basophils being attained at 9 hours.

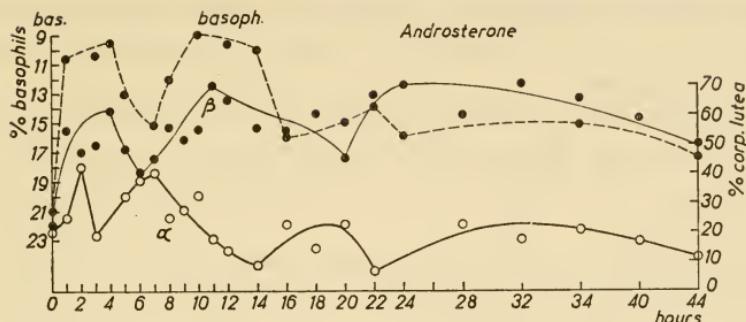


Fig. 47. Ratio between the quantity of basophile pituitary cells and corpora lutea in the  $\alpha$  and  $\beta$  stages after administration of androsterone.

This extrusion was followed again by restitution, which was studied for 10 hours. Corresponding to the rapid fall in the hypophysis curve, there was a reciprocal rise in the curve representing the phases of the corpora lutea. When this increase was completed, transformation into the  $\alpha$ -, and then into the  $\beta$ -stage continued, so that the four successive maxima and minima are shown in the respective curves.

While in the ovary the different phases of the corpus luteum succeed one another there is further secretion of hormone by the hypophysis. The restitution phase, after administration of progesterone, last  $1\frac{3}{4}$  hours; after androsterone (vide Fig. 47), 3 hours; after estradiol (vide Fig. 48), 7 hours. After this first restitution the hypophysis is filled again sufficiently with luteinising hormone to enable the

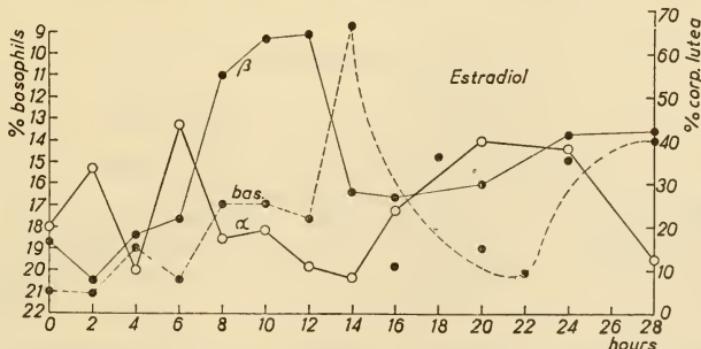


Fig. 48. Ratio between the quantity of basophile pituitary cells and corpora lutea in the  $\alpha$  and  $\beta$  stages after administration of estradiol.

exogenous and still present stimulus to cause a second extrusion. The hypophysis curve, therefore, falls again, representing the  $\beta$ -phase.

In this process the lower limit of the hypophysis curve occurs after the upper limit of the  $\alpha$ -curve, for it is not the extrusion phase itself but the restitution phase following it which is plotted. Thus, the time of the restitution phase of the hypophysis and that of corpus luteum formation partly coincide, so that the maximum of the hypophysis curve corresponds to the minimum of the  $\alpha$ -curve (progesterone, androsterone). This phenomenon points to the correlation between the hypophysis and the ovary.

The rather striking agreement between the maxima suggests a quantitative correlation between the processes. Since the ovary, as the secondary system in this chain-reaction, produces a sufficient number of ova for the formation of corpora lutea, one is justified in saying that a small amount of luteinising hormone produces only a small number of corpora lutea; in other words, the ovary reacts quantitatively to the pituitary hormone.

It is plain, therefore, that response in the bitterling presents the following picture (Fig. 49): the exogenous stimulus (steroid hormone) enters the blood through the gills and affects the hypothalamus. The latter then acts, possibly through nervous channels, on the hypophysis, which, by means of the luteinising hormone stimulates the ovary, to

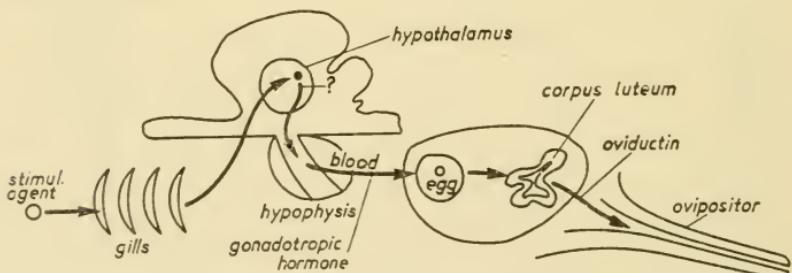


Fig. 49. Diagram showing the concatenation: stimulating agent → hypophysis → ovary → ovipositor.

produce the sexual hormone (oviductin), which causes growth of the ovipositor.

*b. Hypophysectomy and "falling-off" phenomena.*

If the above description of the process is correct, removal of the hypophysis must result in "falling-off" phenomena. This proved to be the case.

*Technique.* After a search for the most suitable method of operation, boring out the hypophysis with a dentist's drill from fishes under narcosis proved to be most effective. The fishes were narcotised with ethylurethane. The palata was sounded with a fine drill until resistance was felt about 1 mm behind the posterior orbital wall, which indicated the bony protuberance of the parasphenoid, protruding into the cavity of the mouth. The hypophysis lies in a bend of this protuberance. The drill was held slanting slightly upwards, drilling being done very carefully, for about 1 mm in this direction. When placed back into fresh water the fish recovered fairly quickly from the effects of narcosis.

All the bitterlings treated were tested with a very active hormone mixture (progesterone, desoxycorticosterone). The majority of the fishes possessed extremely long ovipositors after a 5 hours' test. On histological examination they proved *not* to have been hypophysectomised, the majority having been drilled too far anteriorly. One fish, however, which showed no growth of the ovipositor, was found to have been totally hypophysectomised. Histo-statistical examination showed that the ovaries of those bitterlings in which the hypophysis had not been removed completely, contained numerous corpora lutea (between 42 % and 64 %), whereas to ovary of the hypophysectomised individual contained only a few old remains, not originating from this latest reaction. It is clear, therefore, that (1) *without the pituitary no corpora lutea are produced, and no growth of the ovipositor can therefore result;* (2) the reaction to progesterone and desoxycorticosterone must first *come through the hypophysis*, and does not come direct from the gonads. From this latter fact, which applies, in the bitterling, to all steroids examined, it is clear that the reaction in the fish is the reverse of that in mammals. In mammals, as is well known, the sexual hormone which is administered directly, affects the uterus,

the vagina, the glandula vesicalis, etc. In the bitterling it is only in the hypophysis that administration of a sexual hormone causes a primary reaction, followed by the secretion of gonadotrophic hormone. Thereby corpora lutea are formed, whose hormone (oviductin) stimulates growth of the ovipositor. Oviductin is not identical with progesterone, since the latter does not affect the ovipositor directly, but only, like the estrogenic and androgenic steroids, through the pituitary.

It is evident, therefore, from the hypophysectomy, that the hypophysis is indispensable to the growth of the ovipositor after administration of sexual hormones of the estrane, androstane, and pregnane series. In other words, the reaction of the ovipositor of the bitterling originates in the hypophysis.

## § 2. HOMOLOGY OF THE HYPOPHYSIS-LOBES

### *a. Material.*

After we had succeeded in determining experimentally the position of the gonadotrophic cells of the hypophysis (BRETSCHNEIDER and DUYVENÉ DE WIT, 1939—1940), we were struck by the lack of unanimity, as evidenced in the literature, in the various views regarding the Teleost-hypophysis. Historically the terminology of the structural details of the hypophysis was based upon the mammalian hypophysis. STENDEL (1904), one of the first to give a comparative description of the anatomy of the hypophysis of vertebrates, introduced, for the Teleostia, the term "Uebergangsteil" for a part of the hypophysis which is well defined, but for which he was unable to find a homologue in the mammalian hypophysis. It was CHARIPPER who, in 1937, homologised the different pituitary lobes, and so discarded the rather vague term "Uebergangsteil". With this the desired unity of nomenclature had been established. Our own research group also took this problem in hand, more especially because the gonadotrophic cells were situated in this "Uebergangsteil".

BRETSCHNEIDER and PELSMA made a histological examination of the hypophysis of 19 different vertebrates, i.e., *Petromyzon fluviatilis*; *Polypterus senegalensis*; *Anguilla vulgaris*; *Zoarces viviparus*; *Lophius piscatorius*; *Anomalops graeffii*; *Leuciscus rutilus*; *Rhodeus amarus*; *Carpiodes carpio*; *Lebistes reticulatus*; *Hippocampus antiquorum*; *Triton taeniatus*; *Bufo bufo*; *Xenopus laevis*; *Rana temporaria*; *Seps chalcides*; *Gallus domesticus*; *Serinus canaria* and *Felis domestica*. They arrived at the following conclusions as regards comparative anatomy.

The basic structure of the vertebrate hypophysis shows not three but four lobes, i.e., the lobus tuberalis, lobus anterior, lobus intermedium and lobus posterior, which follow from front to back in this order (Fig. 50A). There is considerable

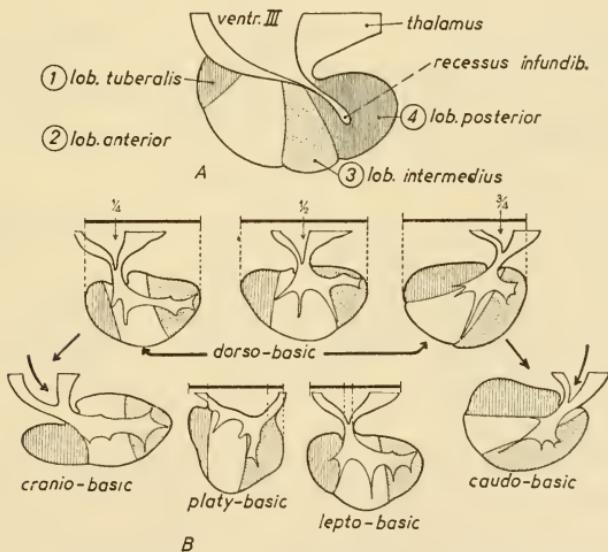


Fig. 50. Scheme of the different pituitary types.

departure from this basic structure, especially in fishes. We have tried to bring some order into this diversity, and have introduced one or two new terms. In this way we were able to distinguish a number of general types having the following characteristics.

*b. Platy- and leptobasia.*

The hypophysis constitutes an aggregate of glandular parts developing from the roof of the mouth the lobus tuberalis, anterior and intermedius, to be called the *adeno-hypophysis*, and one part developing from the brain, namely, the lobus posterior or *neuro-hypophysis*. We can usually recognize a protuberance of the third ventricle through the infundibulum, as recessus infundibuli, in the lobus posterior. This connexion between the hypothalamus and the hypophysis, known as the pituitary stalk, may be present in fishes in the form of a *thin* stem. In this case we say that the hypophysis is leptobasic (Fig. 50B), as in *Rhodeus amarus*, *Carpoides carpio*, *Carassius auratus*, *Lophius piscatorius*, with its long thin stalk is the extreme case. When this connexion, however, is so short that we cannot speak of a genuine stalk, and the hypophysis seems to be attached to the bottom of the hypothalamus, then the hypophysis is *platybasic*, as in *Petromyzon*, *Micropterus*, and others (Fig. 51B). Between these two extremes various transitional forms may be observed with short, broad hypophysis-stems, as in *Leuciscus*, *Zoarces*, *Anguilla*, *Fundulus*, and others.

*c. Position of the hypophysis stem and the hypophysis axis.*

The hypophysis does not always hang at right angles under the hypothalamus, but owing to a shift of its longitudinal axis may assume different positions with respect to the axis of the animal body.

The point at which the stalk enters the body of the hypophysis may be dorsal; the dorsobasal type (Fig. 50B), and as such may be found either in front (*Rhodeus*), in the centre (*Leuciscus*, *Anguilla*, *Fundulus*), or towards the back (*Esox*, *Lebistes*, *Carassius*). Owing to a shift of the longitudinal axis of the hypophysis, through which the hypophysis is directed backwards, the point of entry of the hypophysial stalk may be displaced considerably more towards the front, producing the craniobasal type, as in *Zoarces*, *Carpoides*, and *Lophius*. The cranial or caudal shift of the axis is

also attended by a different orientation of the hypophysis lobes. If we take as our starting point the hypophysis of fishes answering as nearly as possible to the basic design, as, for instance, *Rhodeus* or *Leuciscus*, the lobe tuberalis is most cranially situated. Then follows the lobe anterior, and behind this the lobe intermedium, while the lobe posterior as composed with the higher vertebrates, does not adjoin the hypophysis at the back, but is situated intra-hypophysially. The lobe intermedium is more prominent in fishes than it is in mammals, where the lobe posterior is larger. As the axis of the hypophysis shifts forward the lobe tuberalis becomes more dorsal (*Pseudo pleuronectes*, *Carassius*) and finally comes to lie against the hypothalamus (*Zoarces* or *Carpio*, Fig. 50B), as in mammals, where the lobe tuberalis surrounds the tuber cinereum. In the case of a caudal shift of the

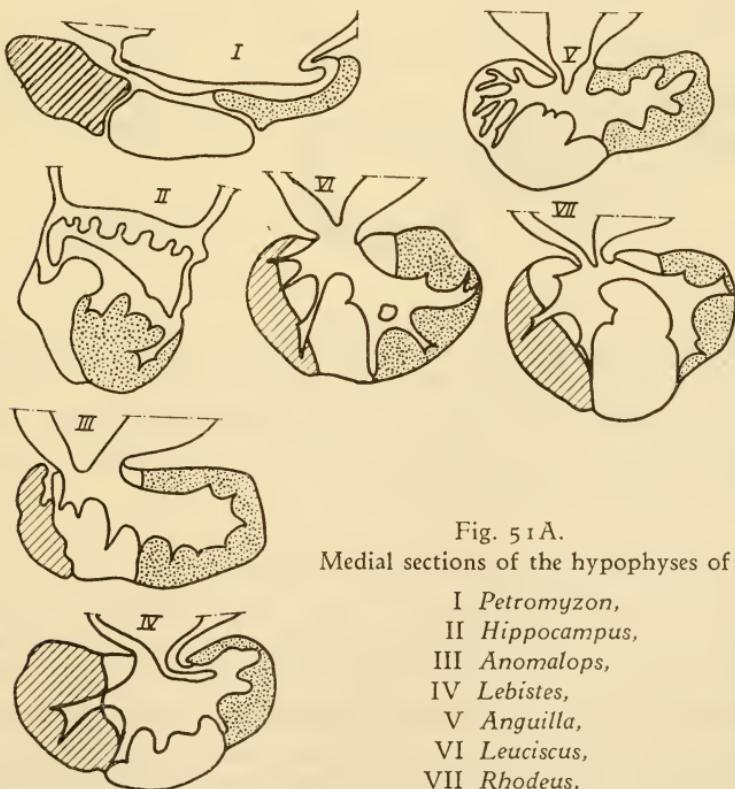


Fig. 51A.  
Medial sections of the hypophyses of:

- I *Petromyzon*,
- II *Hippocampus*,
- III *Anomalops*,
- IV *Lebistes*,
- V *Anguilla*,
- VI *Leuciscus*,
- VII *Rhodeus*,

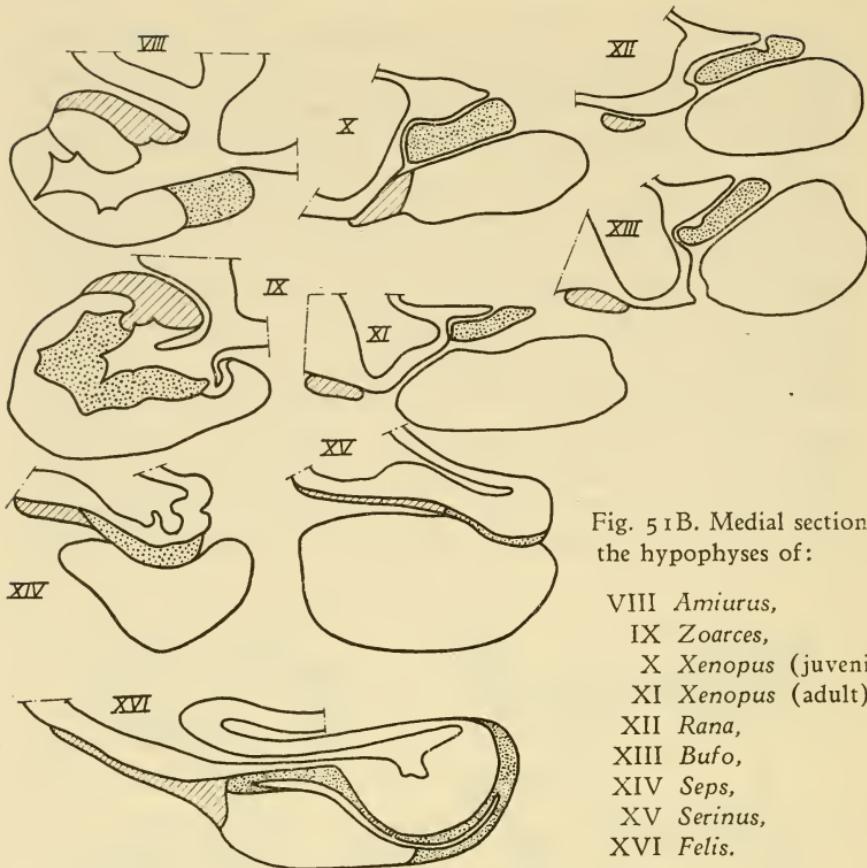


Fig. 51B. Medial sections of the hypophyses of:

- VIII *Amiurus*,
- IX *Zoarces*,
- X *Xenopus* (juvenile),
- XI *Xenopus* (adult),
- XII *Rana*,
- XIII *Bufo*,
- XIV *Seps*,
- XV *Serinus*,
- XVI *Felis*.

axis, the lobus tuberalis in fishes lies against the hypophysial stalk, as, for example, in *Amiurus*. As a result the lobus anterior takes its place on the cranial side of the body of the hypophysis, e.g., in *Zoarces*, thereby conforming to the above-mentioned basic design.

*d. Independence of the lobus tuberalis.*

In *Petromyzon* (Fig. 51A, I) the lobus tuberalis lies as a separate lobe, surrounded by connective tissue, in front of the lobus anterior. In the great majority of bony fishes the lobus tuberalis is directly connected with the lobus anterior, the cells often overlapping one another, although cytological differences in colour and superficial incisions facilitate identifi-

cation of each lobe. In the Teleostei we also find examples in which the lobus tuberalis is connected rather loosely with the lobus anterior, as in *Anomalops* (Fig. 51A, III). Finally, in amphibia the lobus tuberalis is quite separate from the hypophysis, and lies cranially, as a pair of glandular islands, one on each side of the hypophysis (Fig. 51B, X). In young *Xenopus*, however, the lobus tuberalis is still connected with the lobus anterior. In other animals the lobus tuberalis and the lobus anterior form a single lobe. Thus we failed to find an independent lobus tuberalis in *Hippocampus*, *Anguilla*, and *Lophius* (Fig. 51A, V). These two movements of the lobus tuberalis, resulting in independence and movement upwards, already observed in fishes, are carried still further, as we shall see, in amniotes.

In reptiles and mammals this lobe often consists of loose strands of cells, as in birds, or of a plate whose shape resembles something between a shield and a funnel (*Felis*, Fig. 51B, XVI), surrounding the tuber cinereum. This position formerly caused it to remain unobserved, and led to the familiar but erroneous three-lobe division of the hypophysis.

e. *The topographical interrelation between lobus intermedius and lobus posterior.*

There is a constant topographic relation between the lobus intermedius and the lobus posterior. In fishes, with their relatively large lobus intermedius, the main region for the extension of the lobus posterior is situated in the lobus intermedius. However much the lobes may have shifted with respect to one another as a result of a shift of the axis, the lobus intermedius will always be found lying around the body of the lobus posterior. In *Petromyzon* the lobus intermedius is still quite free from the lobus anterior and lobus tuberalis and lies closely against the flat lobus posterior (Fig. 51A, I). In *Zoarces*, with its caudo-basal hypophysial shift, it lies in the centre of the hypophysis (Fig. 51B, IX), both lobes, in this case, being surrounded by the lobus anterior. This is also the case in *Phomoxis*. In amphibia, where the

lobus tuberalis is completely free and the lobus anterior relatively free, there is also a more intimate connection between the lobus intermedius and the lobus posterior than between the lobus intermedius and the lobus anterior (Fig. 51B, X). As is evident from the neurocrinia (p. 71) and the blood supply (p. 62), there is not only a topographical, but also a functional relation between the two lobes. Thus, for example, the lobus intermedius cells in *Anomalops* are placed like a thin layer of epithelium upon the voluminous, richly vascular body of the lobus posterior.

### § 3. THE BLOOD SUPPLY OF THE HYPOPHYSIS IN RHODEUS AMARUS

Since the hypophysis both prepares and receives chemical stimulating substances, an investigation into the blood supply seemed important, the more so because, so far as fishes are concerned, we have as yet no knowledge bearing upon this question. From serial sections, three of which were taken at right angles with each other, DUDOK DE WIT and BRETSCHNEIDER have reconstructed the circulatory system of the hypophysis of the bitterling.

#### a. Position of the hypophysis.

As may be seen from Fig. 52, the narrow stalk of the hypophysis pierces the dural "diaphragm", which lies stretched out in an opening of the primordial cranium. The tunnel-like space found below this is the myodome of the cranium, in which the hypophysis is freely suspended. The extrinsic eye muscles and the bloodvessels lie in the fat and the connective tissue. At the back, the myodome is closed only at the level of the auditory capsule and in front by a thick connective tissue septum.

#### b. The arterial blood supply.

The hypophysis receives arterial blood from the two internal carotids (a), which arise from an open circulus cephalicus.

Immediately behind the branching-off of the arteria orbitalis (e) the carotid artery presents an appearance

reminding one strongly of the glomus caroticus (h), for from this bloodvessels numerous arterioles arise which wind themselves over the entire artery, and, after a short course, are taken up in a vein (m). Some of these arterioles break, as arteriae nutriciae hypophyseos through this connective tissue septum, penetrating into the lobus tuberalis (g). As a second source of arterial blood supply there are bloodvessels which originate in the arteria cephalica (k), and are situated at the front of the thalamus opticus, running through the stalk into the hypophysis. These are the arteriae infundibularis superficiales and internae. Immediately before entering the stalk of the hypophysis the superficial artery suddenly divides into a ball of capillaries, which shortly after are gathered again into a few arterioles, running through the stalk towards the lobus posterior. This artery, therefore, which lies in the dura, but remains close to the surface of the thalamus, constitutes a kind of vascular network, whose significance has not thus far been elucidated, but from whose position, immediately in front of the hypophysial stalk, we may conclude that it bears some relation to the hypophysis. From the caudal part of the thalamus, also an artery, the arteria infundibularis posterior descends through the stalk at the back, penetrating into the lobus posterior. DUDOK DE WIT found, at the root of the arteria carotis interna and in many other vessels of the head, firmly built, funnel-shaped rings facing the bloodstream at their narrow end, and consisting of radially-arranged elastic cells covered by endothelium. KEIBEL (1926) found these rings also in *Cyclostomes* and higher fishes, and is of the opinion that they serve only to catch the rapidly streaming blood in the centre. In view of the narrowed entrance, however, it seems to us that their effect, on the contrary, would rather be to check the bloodstream.

c. *The perihypophysial blood-sinus (Fig. 52 and 53).*

Between the lobus intermedius and the lobus anterior a broad, short vena afferens leaves the hypophysis dorsally (Fig. 52). Thus the perihypophysial bloodsinus provides another source

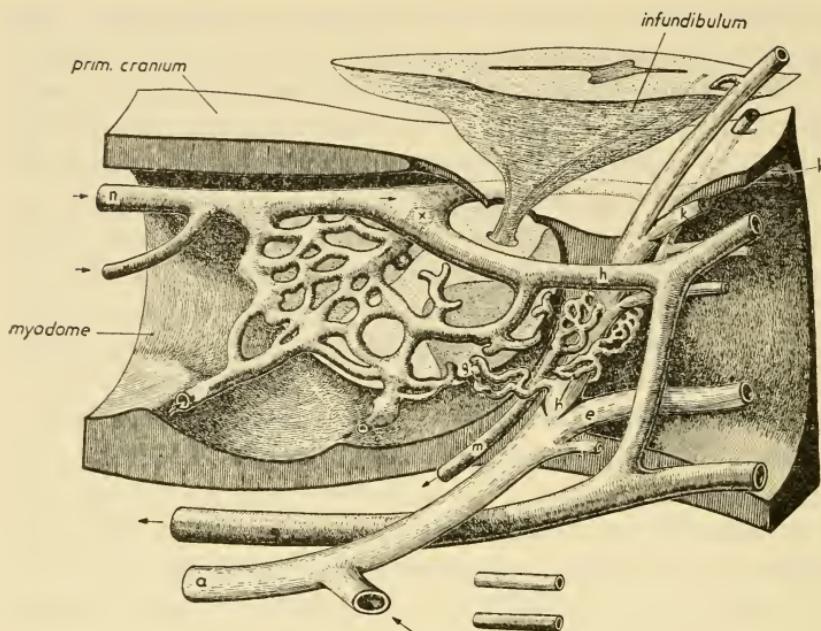
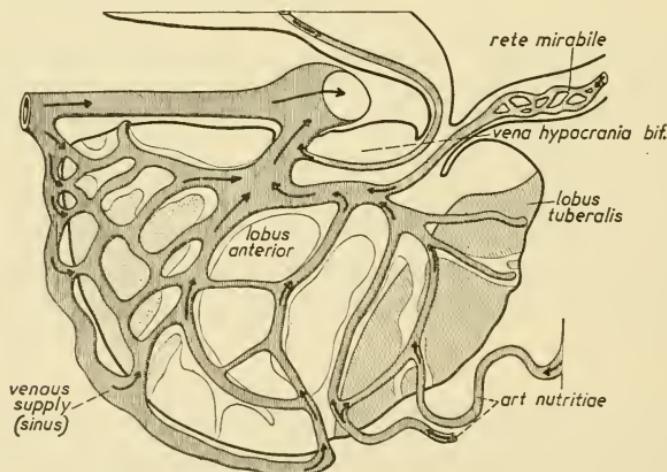


Fig. 52. The blood supply to the hypophysis in *Rhodeus amarus*:

- a. arteria carotis,
- b. arteria orbitalis,
- g. arteriae nutriciae,
- h. glomus caroticus,
- k. arteria cephalica,
- m. vena glomus caroticus,
- n. vena hypocranica bifida,
- x. vena afferens hypophyscos.



Intrahypophyseal blood supply

Fig. 53. Intrahypophyseal blood supply in *Rhodeus amarus*.

of blood for the pituitary. It covers the lobus intermedius and the lobus anterior with anastomosing vessels, penetrating the hypophysis in several places. This blood comes from a number of veins, which join the sinus (Fig. 52 and 53). A single vena hypocranica bifida (n) running through the entire length of the myodome is the chief source of supply of this sinus, and before it splits into two branches which run around the stalk, incorporates the vena afferens hypophyseos (Fig. 52x). The intrahypophysial course of the blood-vessels is characterized by a rich anastomosing system of vessels in the pars intermedia and posterior, whereas the lobus anterior and tuberalis are less plentifully supplied with blood (Fig. 53). The blood of these capillaries soaks the glandular cells and is collected in the lobus posterior into larger veins, which finally unite to form the vena afferens. The arterial blood, from the stalk and the carotis, eventually finds its way into this intrahypophysial system of vessels, so that the blood received leaves the gland again through the large vena afferens. The course of the bloodvessels points in every respect to the existence of a venous system by which a slow bloodstream supplies a large tissue area, carrying with it the substances needed for the production of hormones and gradually charging itself with the latter. Other things pointing to the existence of this slow current are the marked convolution of the arteria nutricia, and the funnel-shaped valves in the carotids, and also, perhaps, the vascular network in the arteria infundibularis.

#### § 4. THE INNERVATION OF THE BITTERLING HYPOPHYSIS

##### a. *Discussion.*

Several facts indicate that the hypophysis is directed from the brain. Changes following section of the hypophysial stalk in animals show that the hypophysis receives nervous stimuli from the hypothalamus. Excitement of the hypophysis to greater activity in the spring is caused, among other things, by changes in illumination and temperature. The condition necessary to display and mating is, as MELTZER has shown, the presence of a mussel. There is therefore no doubt that

nervous stimuli operate upon the sexual-endocrine organization. In our experiments with hormones added to the water we accordingly assumed that these hormones, after being taken in through the gills, reached a nervous centre via the blood, and only thence stimulated the hypophysis to the secretion of gonadotrophic hormones. As the restitution of ova is dependent upon and proportionate to the quantity of corpus luteum hormone produced, we suspected that this hormone is related to the nervous system which then stimulates the hypophysis via the brain. On the basis of these considerations we also studied the innervation of the hypophysis in the bitterling. BRETSCHNEIDER observed this innervation from serial sections after silver-impregnation of both the brain and the hypophysis.

*b. Central tracks (Fig. 54A).*

The principal nerves, such as the tractus praethalamo-saccularis et hypophyseos, emerge from the hypothalamus at the front, through the hypophysial stalk, and may be followed

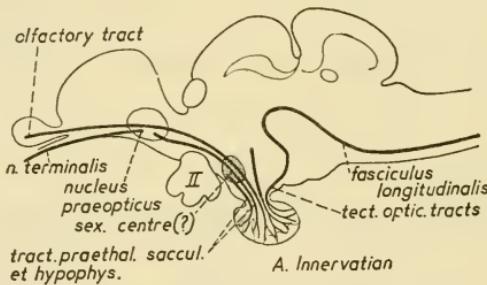


Fig. 54A. Innervation of the hypophysis in *Rhodeus amarus*; central tracts.

from the nucleus preopticus (in which the nervus terminalis ends). The nerves which penetrate the stalk proximally originate in the olfactory tract. This runs dorsally and laterally from the nucleus preopticus to the corpus striatum, and, from here, as tractus striothalamicus et hypothalamicus, reaches the hypophysis. Shorter tracts penetrate the caudal side of the stalk and may be followed as far as the fasciculus

longitudinalis, thus forming a connexion with the medulla. Nerves entering the stalk laterally from the lobi inferiores of the hypothalamus can be followed for a brief distance only; they, however, connect up with tracts running upwards towards the tectum opticum.

c. *Central autonomic centre (Fig. 54 B.)*

HOLMGREN (1920) and SCHARRER (1936) found in the brain of amniotes, central autonomic nuclei, whose ganglion

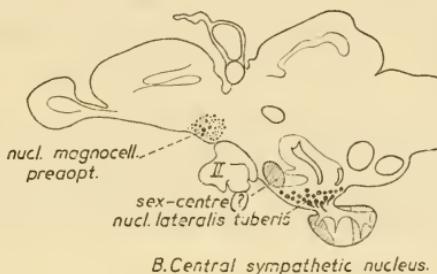


Fig. 54B. Innervation of the hypophysis in *Rhodeus amarus*; central autonomic centre.

cells had changed into secreting cells, and might therefore have a neurocrinal action. In the neighbourhood of the hypophysis there is such a nucleus, the nucleus lateralis tuberis. BRETSCHNEIDER found this nucleus also in the bitterling. It extends in the form of a row of very large cells on either side of the hypophysial stalk. As SCHARRER observed, these nuclei contain large cells of various shapes. There is no indication, however, of any neurocrine function in the bitterling, and in none of our preparations from animals at any season or after any experiment could we find any indication of the production of secretions. On the contrary, we found thick processes coming from these large cells and penetrating into the pituitary stalk, there to join other nerves of more remote origin. These cells also contain NISSL-substance and are clearly nerve cells.

In front of the nucleus lateralis tuberis is a group of cells from which the tracts passing to the hypophysis appear to

originate, and through which pass other tracts, coming from the nucleus praopticus. Here, presumably, is the sexual centre we were seeking; or, at any rate, a nucleus which, together with the nucleus lateralis tuberis, is in direct connexion with the hypophysis.

*d. Intrahypophysial nerves.*

Within the pituitary the nerve fibres lose themselves in the numerous branches of the lobus posterior, becoming embedded in the glia. They either run straight, bend, or turn back in a loop, and emerge from the lobus posterior in a winding course, forming a network around the glandular cell islands (Fig. 55). The local widenings and small loops may be places of contact with the gland-cells. The strongest innervation is that of the lobus anterior; the poorest, of the lobus tuberalis. Upon impregnation with silver, pituicytes appear in the lobus intermedius, with short, impregnated offshoots

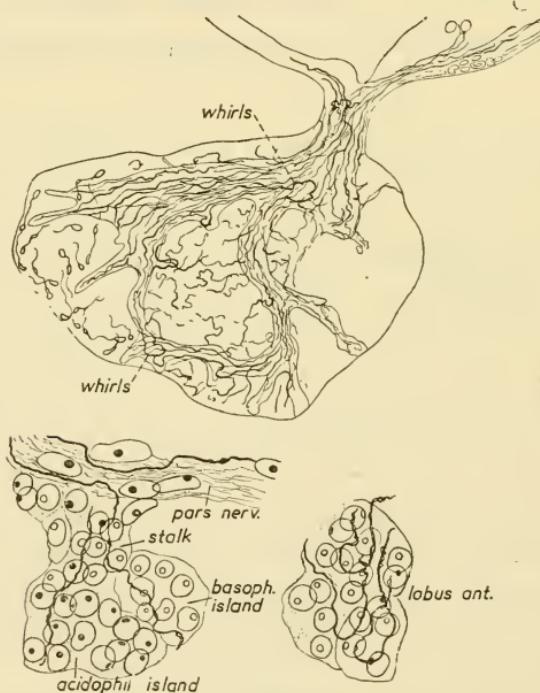


Fig. 55. Intrahypophysial nerves in *Rhodeus amarus*.

having thickened parts, and generally closely connected with similar cells. It is not known whether these are genuine descendants from ganglion cells forming an intramural nervous system, or glia-derivatives, and up to the present their significance is not clear. It is interesting that the lobus anterior, the seat of the gonadotrophic cells, is in direct nervous connexion with the principal tracts coming from the hypothalamus. It would seem reasonable, therefore, to assume the existence of a nervous regulation of the hypophysis in fishes.

#### § 5. THE HAEMO- AND NEUROCRINIA OF THE HYPOPHYSIS OF THE BITTERLING

BRETSCHNEIDER (*vide* p. 49) has shown that the acidophil stage constitutes the phase of formation of the gonadotrophic secretions and the basophil stage the phase of their liberation. He was the first to discover this in the bitterling. In an endeavour to find out more about the activity of the hypophysis we have studied the haemocrine secretion of the lobus anterior and the neurocrine secretion of the lobus intermedius. Whereas BRETSCHNEIDER examined the activity of the lobus anterior during the action of steroid hormones, VAN IERSEL was able to follow this activity under natural conditions and during the whole of the annual cycle, as well as during oviposition, by measuring the sum of all basophil cell islands in the entire gonadotrophic zone (either planimetrically or by means of a photo-electric cell), and calculating the percentage of the total superficial area of the lobus anterior which they occupy. BRETSCHNEIDER found, for four steroid hormones examined, secretion of gonadotrophic hormones, which fluctuated between 15 minutes with progesterone and 2 hours with luteidin (a substance found by means of the ovipositor test, in human urine; *vide* p. 34) followed by a restitution period of varying duration. The action of these haemocrine gonadotrophic hormones may be measured by the degree of luteinisation in the ovary, and by their effect on the ovipositor. The rate of secretion was slower under natural conditions during oviposition. VAN IERSEL found it to be slow

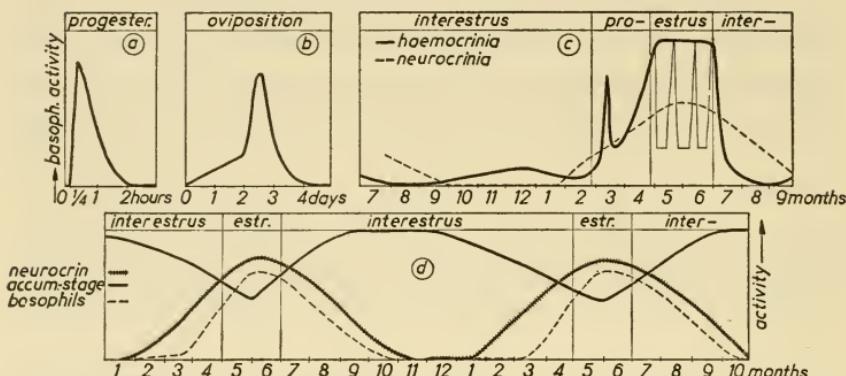


Fig. 56. The relation between haemocrinia and neurocrinia during the cycle in *Rhodeus amarus*.

during the 2 days before oviposition but rising rapidly during that process. There followed an equally rapid restitution (Fig. 56b).

a. During oviposition induced by natural stimuli, there is a less protracted secretion than during oviposition induced by hormones from other species. It is still, however, a relatively rapid process. This is characteristic both of nervous regulation and of haemocrine secretion. Determination of this haemocrine secretion of the lobus anterior throughout the year shows a steady minimum rate in inter-estrus (from September to February), and a high rate in pro-estrus (March), while during estrus, coinciding with every oviposition, the maximum is reached. After this, secretion falls again rapidly to the inter-estrus rate (Fig. 56c).

b. *The transport of hormones through the lobus posterior.*

The secretion from the lobus tuberalis passes exclusively into the blood. That from the lobus anterior is only partly absorbed by the blood. The rest, together with the hormone from the lobus intermedius, passes in the form of a "colloid" through the lobus posterior to the brain. The tissue of the lobus posterior consists mainly of glia, forming a system of fibrous structure running from the thalamus, through the stalk into the hypophysis, passing through all branches of

the lobe and a considerable part of the glandular tissue (Fig. 57). Its parallel fibrous construction suggests that the action of the lobus posterior may be compared to that of the wick of a lamp, the incration being sucked through the stalk to the hypothalamus. The hormone from the lobus intermedium passes in the form of droplets or granules into the lobus posterior. Near the hypophysial stalk the droplets flow together to form larger drops, usually oblong in shape (Fig. 57). VAN IERSEL gave a rough estimate of the amount of

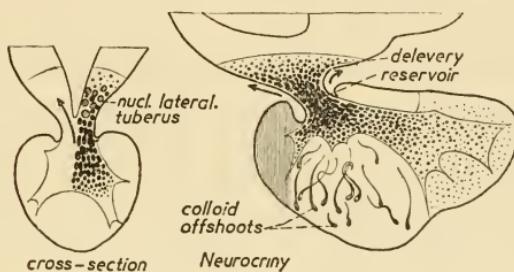


Fig. 57. Neurocrinia in *Rhodeus amarus*.

neurocrinia of the lobus intermedium over a period of twelve months and concluded that in winter the glandular cells contain a maximum amount of incration, whereas in spring and summer they contain less (Fig. 56d). This corresponds with the active phase of the lobus intermedium when the cells lose their marked chromophilia and turn a lighter colour, and also with the time when the lobus posterior becomes filled with colloid. This rough calculation indicated that the neurocrine secretion reaches its maximum at estrus and extends over a fairly long period, being more protracted than the haemocrine secretion of the lobus anterior (Fig. 56c). Especially in spring, but sometimes also in our experiments made during the winter, extra colloid formation takes place, for which we have so far failed to find a satisfactory explanation. From the fairly regular appearance of this phenomenon in the spring, however, we may assume that there cannot be anything abnormal about its occurrence. In a large part of the acidophil tissue the colloid appears at the cellapex in the form

of a long continuous strip, which often runs through the entire hypophysis via the glia tissue and terminates in the stalk. It is as if the secretion were pressed out of the cell in a continuous stream passing upwards amongst the glia fibres. Indeed, large acidophil cells can be seen which push out their incration in the form of threads joined together. In addition, smaller acidophil cells show this abundant production of colloid. The strands sometimes form themselves into spirals in the glia. (Fig. 57).

In normal animals in spring, we found hypophyses which were almost full of such strands of colloid, and whose stalks had been turned into one compact mass of hyaline secretion. We cannot confirm the statement that these secretions, which are carried through the lobus posterior to the brain, reach the third ventricle. We did not find this hyaline incration either in the small recessus infundibularis or in the infundibulum.

From the relatively small quantities passing through the thin stalk at a given moment and available to be absorbed immediately we may conclude that the lobus posterior serves as a reservoir, which may explain the formation of the strings of colloid mentioned above, as a result of internal pressure. The stalk passage, therefore regulates the gradual emission of pituitary hormones in colloid form. We do not yet know which pituitary hormones are secreted as a "colloid", how they enter the main circulation, or reach their destination. The path taken by the substance, however, is as follows (Fig. 57). The mass forms itself into drops in the stalk, and moves like a small stream to the front of the hypothalamus between the capillary system referred to above and the large ganglion cells. A smaller stream flows behind the hypothalamus towards the nucleus lateralis tuberis, between those parts of it that run on either side of the recessus infundibularis. Here the colloid spreads through the interstices and the drops become smaller and finally disappear. The region over which the substance spreads extends to a line dorsal to the nucleus lateralis tuberis, but none is observed beyond this level. The hormone, therefore, enters into direct contact with the ganglion cells, which justifies our calling it a *neurocrine secretion*.

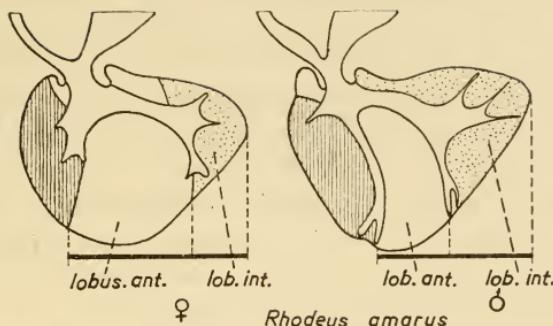


Fig. 58. The pituitary in *Rhodeus amarus* ♀ and ♂.

Our experiments show that the haemocrine secretion occurs in the lobus anterior all the year round, while the colloid secretion from this lobe is added to it only in spring, or very sporadically in winter. There is no doubt that this neurocrine secretion of hormones near the brain has some connexion with a nerve centre in the central nervous system. We are inclined to think that the lobus intermedius is connected with the chromatophores, and we know that, in teleostean fishes, the chromatophores are regulated both nervously and hormonally. Since colour pattern and adaptation to surroundings play an important part in the life of fishes, the marked development of this lobe may thereby be explained. Such an explanation is also indicated by the fact that in the male bitterling, where nuptial characters are well-developed, the lobus intermedius is larger than in the female whereas in the latter the lobus anterior, with its extensive gonadotrophic zone, is more strongly developed than in the male (Fig. 58).

#### SUMMARY (Figs. 59 and 60).

1. The simple construction of the hypophysis of the bitterling makes it a suitable object of study for the quantitative and qualitative determination of the formation and secretion of the gonadotrophic hormone as a result of exogenous stimuli.

Without the hypophysis, growth of the ovipositor cannot take place. The hormone from the hypophysis does not affect the ovipositor directly, but acts on the ovary, resulting in the formation of corpora lutea, and secretion of the hormone (oviductin), which stimulates growth of the ovipositor (Fig. 49). Oviductin is not identical with progesterone. The formation of the gonadotrophic hormone takes place in the "gonadotrophic zone", which is situated in the pars anterior, and the process is rhythmical, as indicated by the regular alternation of acidophilia to basophilia.

2. In accordance with the findings of CHARIPPER (1937) the hypophysis of the fish possesses the four familiar lobes (Fig. 59A), and thus conforms to the general basic plan of

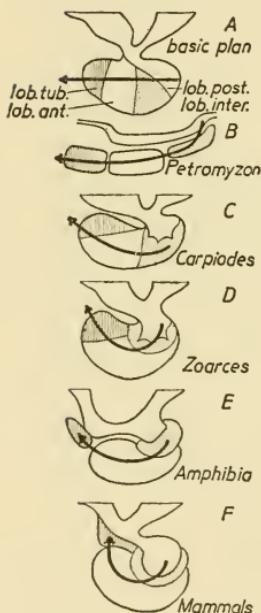


Fig. 59. Schematic reduction of the different pituitary types found in vertebrates.

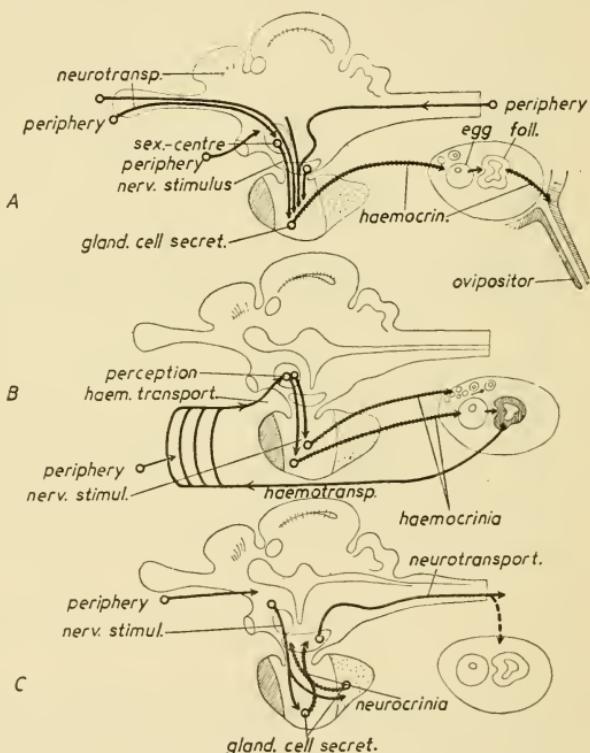


Fig. 60. Diagrams showing haemo- and neurocrinia in *Rhodeus amarus*.

the vertebrate pituitary. The basic plan of the hypophysis of Sauropsida and mammals (*Gallus*, *Felis*) may be inferred from the turning of the hypophysis axis (*Carpoides* and *Zoarcæ*) and a certain independence of the lobus tuberalis (*Xenopus* and *Bufo*). The term "Uebergangsteil" (transitory part) should be dropped, and replaced by the term lobus anterior. It appears from the position of the gonadotrophic glandular cells, described on p. 56, that, in fishes, these are present also in the lobus anterior, and that, therefore, the hypophysis in fishes does not deviate from the structural plan as known at present.

3. From an investigation of the blood supply, the innervation, and the haemo- and neurocrinia of the hypophysis of the bitterling we are now able to form the following provisional picture (Fig. 6oA).

a. There is a connexion between the periphery (i.e., the outside world) and the hypophysis, through tracts running from the senses to the hypothalamus and thence to the hypophysis. Here, the glandular cells are stimulated to produce their hormone which passes to the effector (the ovary) via the blood.

b. When steroid hormones are added to the aquarium water, the stimulus passes through the blood vessels of the gills and probably reaches a centre in the brain, whence a nervous stimulus then acts on the glandular cells in the hypophysis. These glandular cells send their hormones through the blood to the effector, the ovary. The stimulus described on p. 26, from the ovary to the hypophysis, will also be received in the brain, reaching the hypophysis via nervous channels (Fig. 6oB). In both cases, therefore, there is a nervous path to the hypophysis, and a blood path to the effector. As is evident from experiments and the observation of the normal course of events during the year, secretion into the blood is completed rapidly, taking not more than one day.

c. By a third channel (Fig. 6oC) the hypophysis receives from the brain a nervous stimulus, after which the glandular

cells pass their product back to the brain, which in turn influences the effectors (the chromatophores, and perhaps also the gonad). This influence on the brain is much slower and more gradual than the transport in the blood. It is evident from all this that the part played by the hypophysis, as "motor of sexuality" (ZONDEK) in the chain of reactions is an extremely complicated and many-sided one, and that, owing to the location of the hypophysis (as an appendix of the hypothalamus), there is close connexion, both nervous and humoral, with the periphery as well as with the central parts of the body.

### III. THE CORPUS LUTEUM AND OTHER FOLLICULAR DERIVATIVES IN VERTEBRATES

#### § 1. THE STRUCTURE OF THE OVARY

In our search for the gland which we believe to be responsible for the growth of the ovipositor, we directed our attention in the first place to the ovary. We then found corpus luteum-

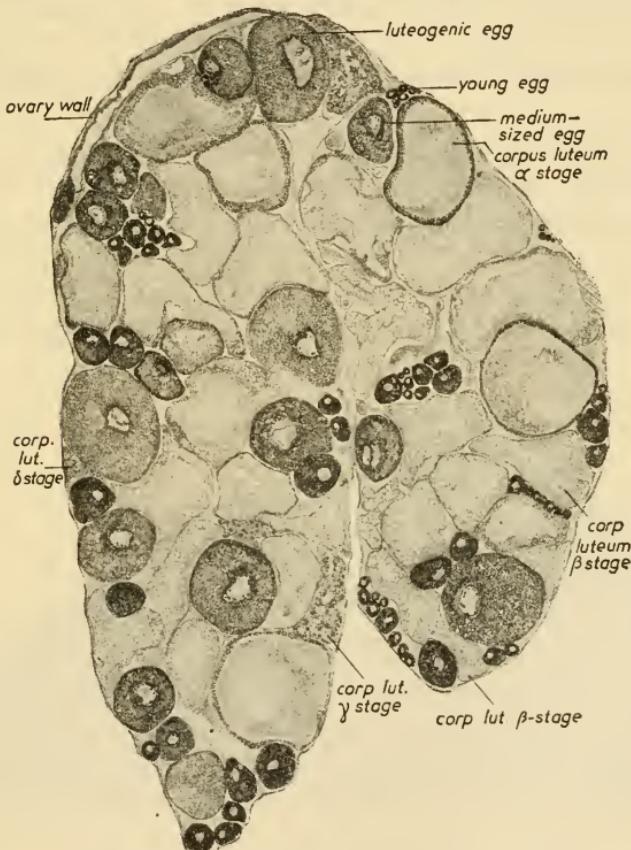


Fig. 61. Transverse section through the ovary of *Rhodeus amarus*. Apart from small, medium-sized and large eggs, all stages in the formation of corpus luteum are seen.

like formations, derived from ovarian follicles. Before examining the origin of these corpora lutea we shall first describe briefly the histology of the ovary and the development of the ova and the follicles.

The ovary of the bitterling is a thin-walled, pocket-shaped, hollow organ. Its surface area is increased by the formation of small pocket-like concavities in the wall (Fig. 61). The wall itself consists of three layers of cells: (1) a firm connective tissue layer, containing blood- and lymphatic vessels, collagen fibres, oblong connective tissue cells with numerous xanthophores and melanophores; (2) the peritoneum, a very thin epithelium upon this connective tissue layer on the outside; (3) a nuclear epithelium covering the connective tissue layer on the inside. The cavity of the ovary is continuous with the oviduct, the epithelium of which contains mucous- and ciliated cells. In this way the ovary, with its numerous egg-pouches, hangs in a space closed off from the abdominal cavity and continues caudally into the ovipositor.

The function of the ovary of the bitterling is (1) generative, by the production of mature ova; (2) regulative, by

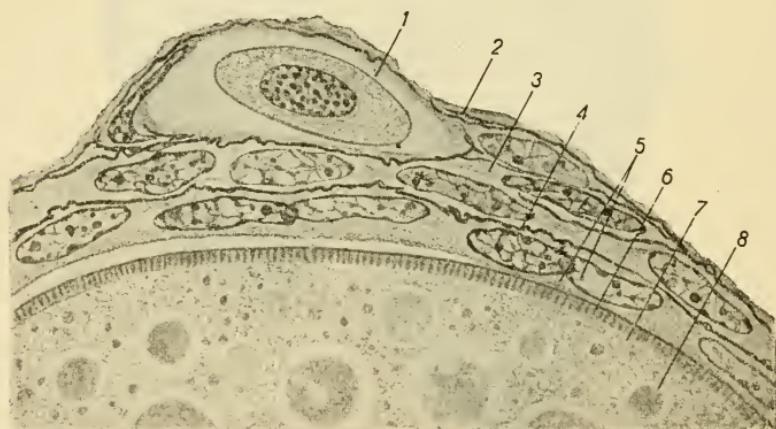


Fig. 62. The follicle of a luteogenic ovum:—

1. capillary,	4. basal membrane,	6. oolemma,
2. theca externa,	5. granulosa,	7. egg-plasm,
3. theca interna,		8. yolk.

the continuous formation of short-lived hormonal glands, and (3) reparative, through the restitution of the different structures according to the losses sustained.

Microscopic investigation of a mature follicle (Fig. 62) reveals the following structures: (1) The ovum is surrounded by a membrane, the *oolemma* (*zona pellucida*). Upon this lies an inner follicular layer, (2) the *granulosa*, consisting of a single layer of cells. The *granulosa* cells hang together by means of very broad plasmodesmas. Outside the *granulosa* there is a wide-meshed layer of cells, (3) the *theca interna*. The cells composing the latter hang together by means of fine plasmodesmas. Between the cells there are spiral-shaped collagenous fibrils, lymphatic cavities and capillaries. Finally, the follicle is enveloped by (4) the *theca externa*, consisting of spool-shaped cells.

The ova develop in two different places in the ovary, i.e., caudally and cranially. These are the oogenetic fields, where a rapid production of ova takes place at certain times. In the outer zones of these oogenetic fields the following processes may be observed (Fig. 63). The nuclear epithelium, consisting of a single layer, has quite a uniform appearance outside the oogenetic zone. Nearer the boundary of the field the epithelium is more active; it becomes raised, and the nuclei swell into small cysts. Thereupon follows the first visible differentiation: some of the cells enlarge, their nuclei become spherical in shape, possess a large, nucleolous and linin network with numerous chromatin islands; the cytoplasm also dilates, numerous vacuoles and granula appear. These enlarged cells later become the ova. Simultaneously, the neighbouring cells undergo a different change. Their nuclei become extended, and acquire a fine network of linin threads and only little chromatin. Through the pressure of the neighbouring growing cells both the nucleus and the cytoplasm become flattened, and the cells place themselves like bonnets against the growing cells. These cells also seem to have a certain mobility, for they sometimes move to the base of the ovum. These attendant cells become the future *granulosa* cells of the follicle. On close examination of the boundary zone an alternating develop-

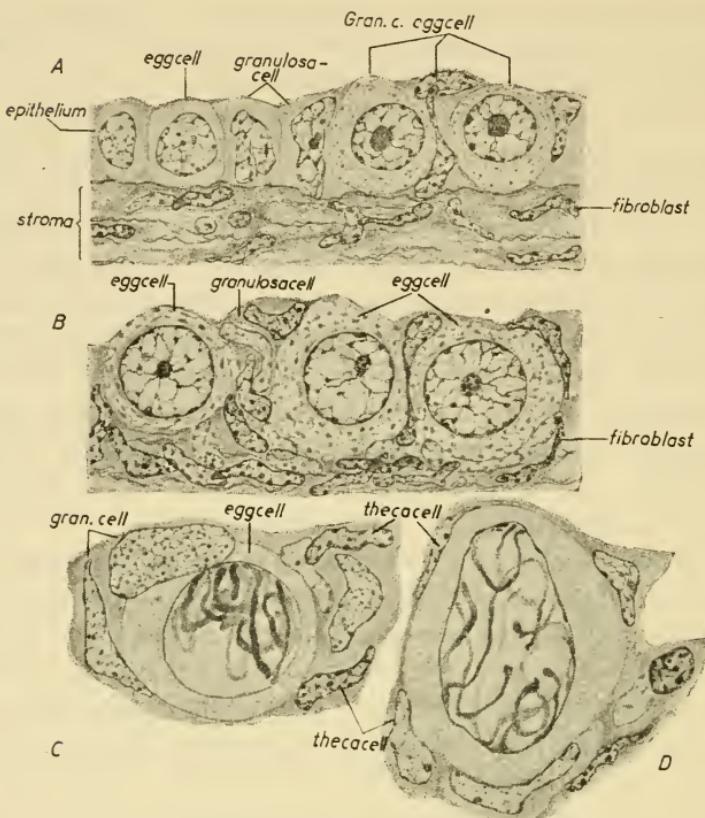


Fig. 63. The genesis of egg and follicle in the nuclear epithelium: (a) on the border of a neogenic field; (b) further advanced stage of development in the centre of a neogenic field; (c) and (d) joining of the cellular material into a follicle.

ment may be observed: an ovum is formed first, and after this a primary granulosa cell (Fig. 63a, b).

With this the first step is taken towards the development of the two most important follicular components, i.e., the ovum and the granulosa cell. Before the follicles are formed, there is first a *proliferation period*. The ovum grows, and forms, together with the 2—3 granulosa cells, a button-like elevation, which emerges from the nuclear epithelium. At this stage a number of fibroblasts are liberated from the connective tissue layer of the ovary wall and arrange themselves around this raised group of cells, producing fine collagen fibrils.

These cells later become *theca cells*. In these cell-islands there is active cell-division resulting in the production of oocytes which develop into ova. Not until then do the three components, i.e. the ovum, the granulosa and the theca, join together to form a follicle. Some of the granulosa cells surround the ovum, and upon this simple granulosa layer the theca cells are laid down. Thus, the follicle has come into existence. Figs. 63 c and d show two different stages in the development of the follicle: the proliferation stage and the completed follicle.

Although the notion "follicle" is used in connexion with the mammalian ovary, and was originally conditional upon the presence of a follicular cavity, we propose to retain it in the present context, although no cavity is formed in the follicles of the fish. In the primary follicle of mammals, however, there is no cavity, and one might say, therefore, that the ovarian follicle of the fish remains at the primary stage for the duration of its life.

The ovum and the follicular wall grow steadily until maturity. Four different stages in the growth of the ovum may be distinguished.

- a. Small ova; average diameter 100  $\mu$  (10—200  $\mu$ ): ovarian cytoplasm, acidophil; no yolk present.
- b. Medium-sized ova; average diameter 300  $\mu$  (200—500  $\mu$ ): ovarian cytoplasm; basophil; primary yolk formation has started; no oolemma present.
- c. Large ova; diameter between 500 and 1000  $\mu$ ; ovarian cytoplasm, basophil; secondary yolk and oolemma formation begins.
- d. Maturing and mature ova; diameter between 1000 and 1800  $\mu$ . The ovarian cytoplasm is divided into a vegetative part containing the yolk, and an animal part containing the nucleus; a thick oolemma envelops the egg.

## § 2. HISTOGENESIS OF THE CORPORA LUTEA

We have already mentioned that, in our view, it seems as if corpora lutea with a secretory function occur in the ovary of fishes. This is surprising, since up to the present no such appearance of corpora lutea in anamnia has been observed.

The scanty information available concerning the histology of the ovary of the fish occasionally contains references to the corpora lutea found by us. Such formations, however, were believed to arise from *degeneration of the ovum*. BÜHLER (1902), (*Petromyzon* and *Coregonus*), and, more recently, LYNGNES (1937), (*Myxine*) described the fate of follicles remaining behind after ovulation. Both these writers were struck by the fact that it is possible also for non-ovulated follicles to "degenerate", even before the ovum matures. Both take this process to be what is known as "ovular atresia", and find it to be merely the interruption of some process of growth. LYNGNES describes in detail certain forms of "ovular atresia" and is of the opinion that they are caused through damage to ova as a result of the growth of other ova.

These authors did not expect to find a close relationship between the gonads and secondary sexual organs in anamnia, and so did not assume the occurrence of corpora lutea with secretory activity. It was only through the experimental induction of growth in the ovipositor that the hypothesis arose that there might possibly exist a connexion between the ovary and the growth of the ovipositor.

In the bitterling the corpora lutea develop chiefly from the ovarian follicles of the third period of growth (500—1000  $\mu$ ). The process of disintegration of the ovum and of its conversion into the corpus luteum may be divided into four successive stages:—

a.  $\alpha$ -stage. The ovum is attacked by follicular cells, made resorbable, and finally resorbed and phagocytosed.

b.  $\beta$ -stage. In the follicular cells a new substance is formed, the lengthening hormone, oviductin, which is absorbed by the blood.

c.  $\gamma$ -stage. Hormone production ceases when disintegration is completed, the yellow lutein pigment remaining behind, as cellular residue in the follicular tissues.

d.  $\delta$ -stage. The resulting corpus luteum partly atrophies into an irregular heap of cells, the majority of which are differentiated into histiocytes (BRETSCHNEIDER, DUYVENÉ DE WIT, 1941).

We will now examine the four stages separately in more detail.

a. *The  $\alpha$ -stage.* Two factors indicate the transition from

the normal follicle to the beginning of the formation of a corpus luteum: (a) the ovum itself, in which certain changes take place and (b) the granulosa cells which are distinguishable from follicle cells of the same age and/or size.

Disintegration of the ovum occurs in the initial stages of the process. The contents lose water (Fig. 64) and contract, causing the oolemma to become wrinkled. Then follows a very obvious change with separation of the yolk granules from the more liquid plasm. Finally the nucleus disintegrates, bursts, and mixes with the cytoplasm. Sometimes this happens so suddenly that we might say the nucleus "explodes" (Fig. 64).

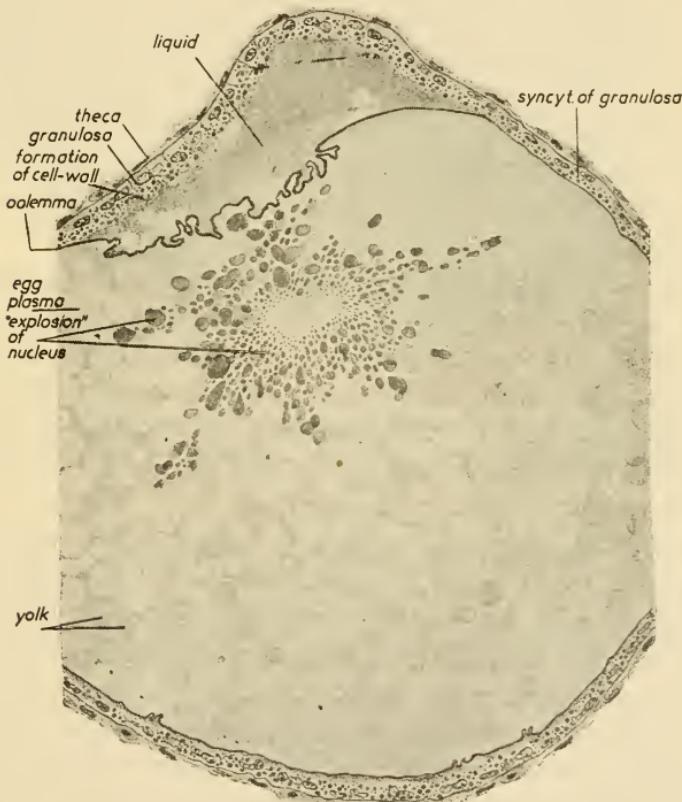


Fig. 64. Luteogenic egg-follicle at the beginning of the formation of a corpus luteum: beginning of the  $\alpha$ -stage.

*Changes in the granulosa cells* occur simultaneously with those in the ovum. The cells rapidly increase in size. The originally narrow, flat nuclei become larger, more oval in shape and more transparent, and nucleoli make their appearance. The cytoplasm of the syncytium absorbs liquid, which causes it to expand. For the time being it remains fairly homogeneous, but when the granulosa cells have changed sufficiently to justify our referring to the formation of corpora lutea, the most typical stage has been reached, namely, the active production of granules within the cytoplasm of the granulosa cells, and the division of the syncytium into single cells. At the same time there is a great increase in the height of these newly-formed cells, and their nuclei become larger and more active. The conversion of the syncytium into single, well-defined granulosa cells points to the fact that a definite function has been acquired. The granules disappear soon after they are formed. They are extruded at the apex of the cell, partly in the form of granules and partly in liquid form, and place themselves between the oolemma and the granulosa.

The oolemma is fairly thick, and is pierced by numerous small pores lying side by side and radially with respect to the surface of the ovum. Viewed from above they present a sieve-like appearance, and may allow the passage of substances from the granulosa cells. Immediately after the appearance of granulosa cell secretion the first changes occur at the surface of the ovum, beneath the oolemma. The round, clearly defined particles of the yolk are liquefied and fuse into irregular drops (Fig. 65). Simultaneously the oolemma itself is changed. The drops appear, showing a distinct transition from acidophil into basophil cells. As a result, breaches occur in the wall between the ovular and the follicular tissue, so that the cytoplasm of the ovum and the granulosa cells now come into immediate contact with each other. These changes probably do not originate in the ovum itself, but are initiated by the secretion from the granulosa cells. The granules of the granulosa act as it were as a "disintegrating ferment"; they

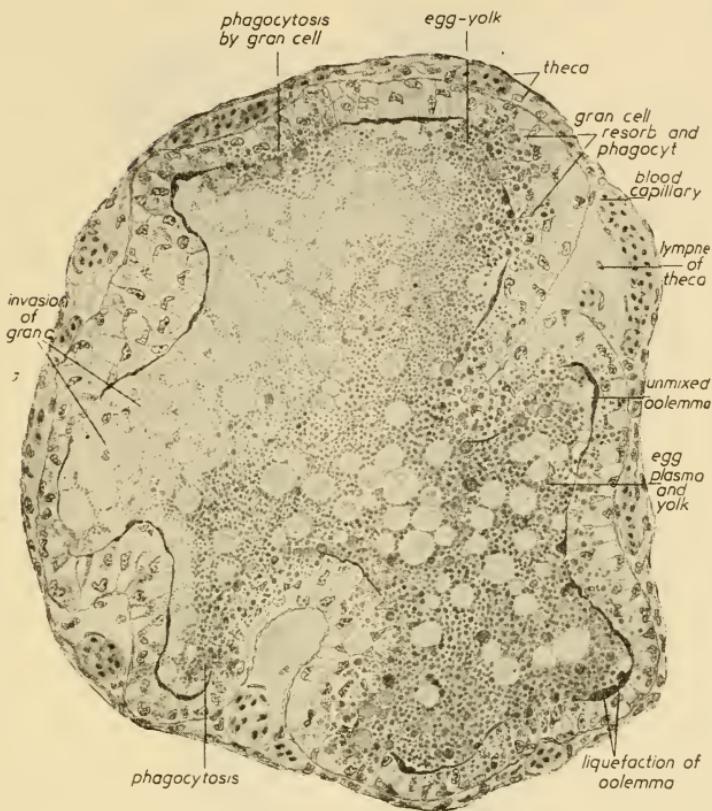


Fig. 65. Advanced stage in the formation of a corpus luteum. Granulosa layer in different stages of resorbent action; phagocytosis of the yolk substance through the granulosa cells. Granulosa cells invading substance of yolk.

dissolve the oolemma and prepare the contents of the ovum for resorption.

Whereas the first function of the granulosa cells is to prepare and secrete a ferment, their subsequent activity proceeds in the opposite direction, i.e., *the absorption of partly liquefied, and partly solid substances from the ovum*. Absorption by the granulosa cells takes place through *resorption* of liquefied ovular substances and *phagocytosis* of solid yolk elements. The cells which have been emptied by extrusion rapidly fill up with yolk particles of different size

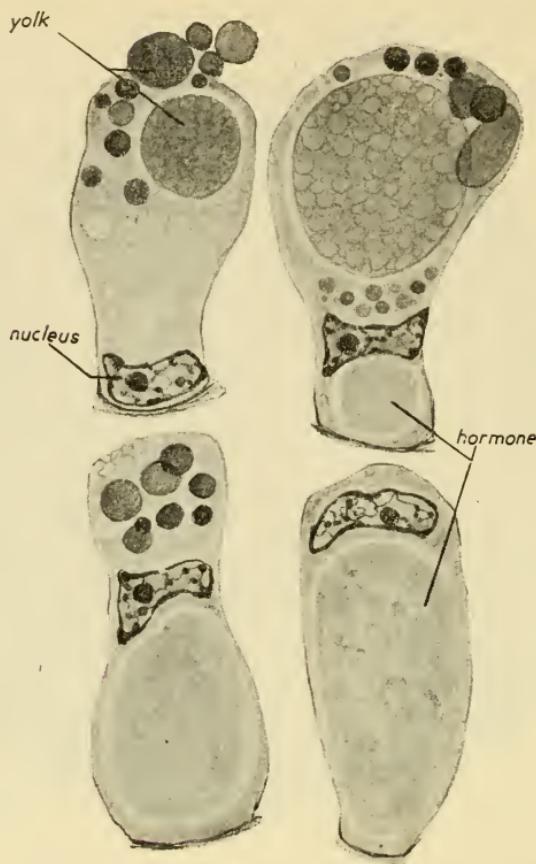


Fig. 66a. Granulosa cells. Apical cell-space containing phagocytosed ovular substances; basal cell space filled with basal vacuole.

(Fig. 65). Even the yolk-granules larger than the original granulosa cells are phagocytised, so that the height of the cells may increase sevenfold. Through resorption and phagocytosis the original contents of the ovum disappear and the follicular wall is deformed under the pressure of the adjacent parts of the ovary.

We shall mark this point as the end of the *a*-stage, whose characteristics are (i) enlargement of the granulosa cells; (ii) conversion of the granulosa syncytium into single cells; (iii) lively production of basophil granules; (iv) extrusion of the liquefied granules, and (v) resorption and phago-

cytosis of the egg contents by the granulosa cells, accompanied by marked increase in the size of the cells.

*b. The  $\beta$ -stage.* This stage is characterized by the conversion of the substances within the cells, and its absorption by the blood. When the cell in question has taken up a certain amount of ovarian substance, the basally-situated cell shifts towards the apex, in such a way that the nucleus (much like the piston of a pump) rises along the cell-wall, thus dividing the high cylindrical cell into two separate parts (Fig. 66a), which differ from each other in structure. The *apical cell-space* still contains the phagocytosed ovarian substances in the form of granules, and also some yolk. In the *basal cell-space*, on the contrary, a small vacuole may at first be observed, filled with a homogeneously coloured, often finely-granulated substance, whose chemical and physical behaviour differs from that of the apical ovarian material. The quantities of material respectively taken up apically and accumulated basally are inversely proportional; the larger the basal vacuole, the smaller the amount of apically admitted material we shall find, and the higher the nucleus will be, until at last the latter is able to reach the apex. Often the granulosa cell is completely filled by a *large basal vacuole*, for which reason we have provisionally called it a "vacuole cell" (Fig. 66a).

Simultaneously with this conversion of substances within the vacuole cells a rapid *cellular division* takes place among the granulosa cells. Only rarely are mitoses found; amitotic division of nuclei, on the other hand, occurs very frequently. After the division of the nucleus, part of the cytoplasm may be seen to split off, with one of the nuclear parts, from the mother-cell. This causes the granulosa layer to increase rapidly in thickness (Fig. 66).

Further, these daughter-cells often become free and *invade the follicular space* (Fig. 66). Both in the mother-cell and in the daughter-cells the incorporation of yolk continues. Further division occurs in those daughter-cells which have invaded the follicular space; they are often found joined to form long strands, or they may form small syncytia with several nuclei (Fig. 66).

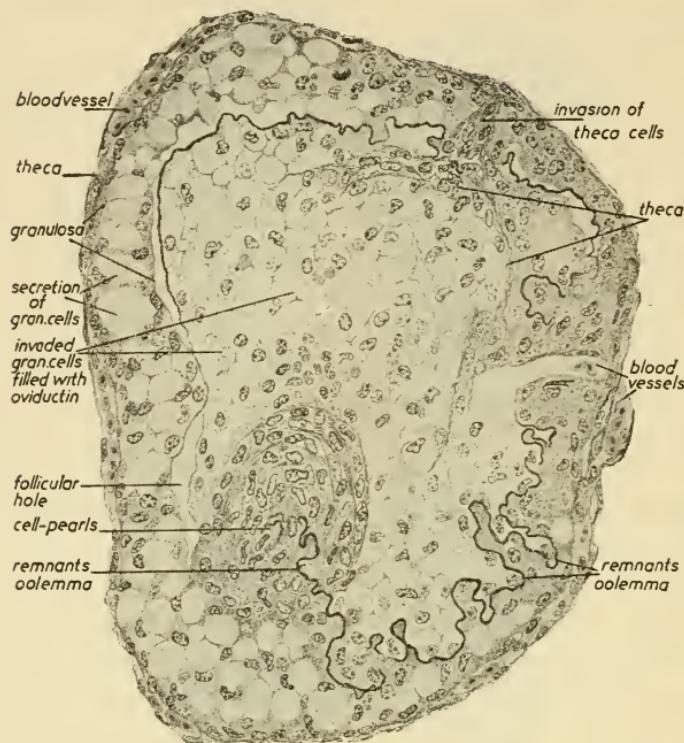


Fig. 66. Corpus luteum,  $\beta$ -stage, ready to secrete; the egg has disappeared, its cavity being filled with granulosa-lutein cells; invasion of theca tissue and bloodvessels.

Meanwhile the granulosa cells have completely incorporated and assimilated the ovular substances. The remaining inner space of the ovum consists of liquid, insofar as it does not still contain granulosa cells (Fig. 66, left). At this stage of development of the corpus luteum all granulosa cells are seen to possess a foamy plasm rich in vacuoles. Apart from a somewhat denser plasm in the neighbourhood of the nucleus, the numerous vacuoles occupy almost the entire cellular space (Fig. 66). They are of different sizes, lie adjoining one another, and are separated by thin walls.

Our cytological investigations, which are not yet completed, have so far shown that in the base of the granulosa cells new substances are formed with the aid of mitochondria

and GOLGI bodies. These new substances arise at first from small albuminous granules at whose surface numerous exceedingly small drops of fat may be found. These albumin-fat systems, which we consider to be the forerunners of the hormonal products, dissolve during the course of the further development of the corpus luteum and finally appear as vacuoles in the cytoplasm of the granulosa cells; thus, their whole appearance is similar to that of the "lutein-cells" of the corpora lutea of mammals.

The further these lutein-cells penetrate into the ovarian space the greater their distance from the blood-vessels and lymph-sacs becomes, and the more difficult the emission of the accumulated hormonal substances. This difficulty arises soon after the separation of the daughter-cells, with the result that these cells become charged with numerous vacuoles and expand considerably. In several places the strongly vasiculised theca breaks through the granulosa and penetrates into the follicular space filled with lutein cells (Fig. 66). The long reel-shaped theca cells mix with the large lutein cells and form—as in the case of the mammalian corpus luteum—a connective tissue framework, around which the hormone-forming cells group themselves. Bloodvessels appear in the theca tissue if the ovary is stimulated (e.g., by injection of an extract from the hypophysis of the carp) and there follows active formation of corpora lutea; then the blood-vessels of the theca and those in the follicular space are filled to capacity, which may lead to large extravasates. Even macroscopically these blood-filled follicles may be distinguished as red blood-points.

Simultaneously with the invasion of the theca cells other granulosa cells, which have not participated in the phagocytosis of the yolk, and possess a dense cytoplasm, penetrate into the corpus luteum space and clear away any oolemma remnants that have not yet been dissolved (Fig. 66, below). The arrival of these cells results in a dense mass of tissue which grows around the oolemma remains in concentric layers. Thereby so-called "cell-pearls" are formed, which finally, by fusion and increase in size, grow into cystoid

formations, the oolemma remnants inside them subsisting for a long time.

The processes so far described lead to the formation of a perfect corpus luteum. Through the transformation of the granulosa cells into lutein-cells and their contact with blood-vessels the active area of the corpus luteum is noticeably enlarged: this implies that the former ovarian content can be transformed into hormone within a short time, and rapidly secreted into the blood.

The  $\beta$ -phase has the following characteristics:—

- (i) assimilation of the ovarian substances already taken into the cytoplasm by the granulosa cells;
- (ii) movement of the nucleus towards the centre of the cell;
- (iii) rapid multiplication of the cells;
- (iv) movement into the ovarian space;
- (v) formation of oviductin in the granulosa cells;
- (vi) presence of numerous bloodvessels in the ovarian space.

c. *The  $\gamma$ -stage.* As soon as the corpus luteum has matured, it begins to disintegrate. Substances still present in the cells are transformed into oviductin. The vacuoles become smaller owing to loss of hormone, and the lutein cells are reduced in size. Eventually the wall of the follicle collapses, and both blood- and lymphvessels gradually disappear. The cytoplasm of the lutein cells grows denser as the vacuoles are lost. The cell content of the theca becomes looser. With this the actual stage of decay has set in.

One of the chief characteristics of this stage is the formation of an intensely *orange-coloured pigment*, inside the lutein cells (Figs. 67 and 68), a property to which the corpus luteum owes its name, a similar pigment being present in the corpora lutea of mammals. As far as our cytological knowledge of the matter goes, the formation of the yellow pigment would seem to be the result of *degeneration* of the granulosa cell cytoplasm; for the entire plasmatic cellular content falls apart into yellowish red granules, which retain their peculiar colour even after the cell has been stained. At this stage only little of the former distinction between granulosa and theca can be seen. All the cells eventually fuse to form a spherical

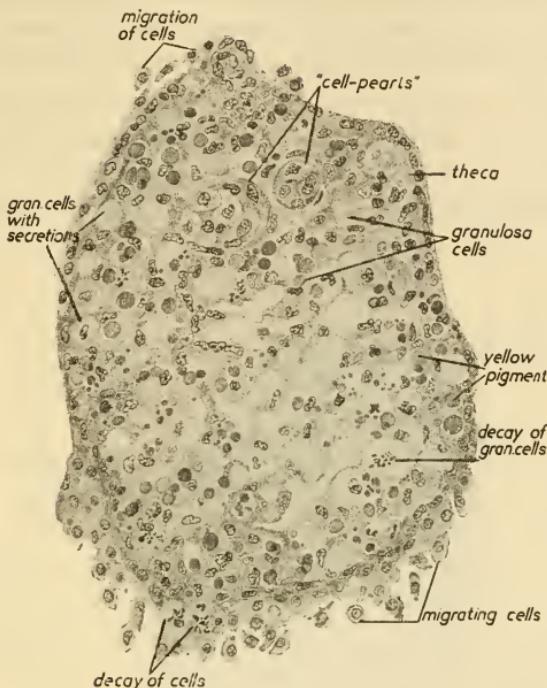


Fig. 67. Corpus luteum,  $\gamma$ -stage. Decay of the already largely inactive corpus luteum; appearance of corpus-luteum pigment in the lutein cells. Loosening and migrating cells; a few, fully secreting granulosa cells.

mass. Only on the extreme edge is a little of the theca, consisting of connective tissue, still recognizable (Figs. 67 and 68).

The cells of the corpus luteum become smaller and fewer with the successive stages of degeneration, and our investigations have shown that in the  $\alpha$ - and  $\beta$ -stages the number of cells increases considerably, whereas with the completion of the  $\beta$ -stage it rapidly decreases to zero.

This decrease in the number of cells takes place in two different ways: by *migration of the cells* and by *necrosis* (Figs. 67 and 68). The migration of the exhausted corpus luteum cells is preceded by a re-differentiation. Owing to the disappearance of the vacuoles during the preceding stage, the cytoplasm becomes denser. The pigment disappears again;

the nuclei become smaller and rounded, and the body of the cell stretches into a reel-like shape. These differentiated cells then show a strong likeness to the stroma cells of the ovary, and leave the mass of cells of the degenerating corpus luteum apparently by amoeboid movement. In long strands, or on a broad front, they then migrate through the other ovarian tissue, joining up with the rest of the ovarian stroma.

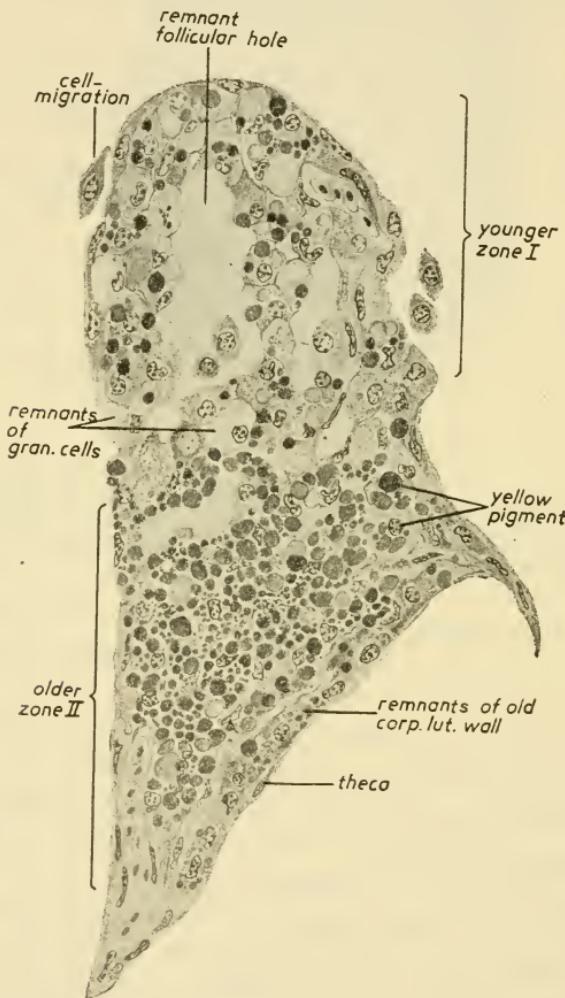


Fig. 68. Corpus luteum,  $\delta$ -stage. Zone 1 is in an earlier phase of development than zone 2; the latter consists as yet only of a conglomeration of yellow-pigmented cells.

The  $\gamma$ -stage has the following characteristics:—

- (i) beginning of the degeneration period;
- (ii) formation of an orange-yellow pigment in the granulosa cells;
- (iii) migration of the cells and necrosis.

*d. The  $\delta$ -stage.* After this spontaneous migration the remaining cells disappear. The nuclei become pycnotic and form long threads; the cytoplasm disintegrates. In the end only a light yellow mass of tissue remains to mark the former presence of a corpus luteum (Fig. 68). With this the span of life, so full of variation, of the corpus luteum in the bitterling is terminated.

As we have seen, the actual secretory glandular tissue in the bitterling, like that of the corpus luteum in mammals, originates in the *granulosa layer*. This is characteristic only of the genuine corpus luteum; for in the case of the atretic follicle or the atretic corpus luteum, the glandular tissue arises in the *theca interna*. Whereas, in mammals, the corpus luteum is formed after ovulation from the follicular wall, corpus luteum formation in the bitterling already begins before ovulation, and the ovum is sacrificed. We would, therefore, give the name "*pre-ovulation corpus luteum*" to the type found in the bitterling, in contradistinction to the "*post-ovulation corpus luteum*" found in Sauropsidans and mammals.

It will be seen later that the pre-ovulation corpus luteum constitutes an integral part of the ovaries of all anamnia, reptiles and birds, and that, in these animals, it also plays an important part in their sexual-endocrine system.

### § 3. THE OVO-FOLLICULAR SYSTEM

As a result of further research it was shown that not only the bitterling but also other non-mammalian vertebrates possess secretory corpora lutea (BRETSCHNEIDER (*Selachii*, *Teleostii*, *Amphibia*, *Reptilia*); VAN EGMOND (*Selachii*); JASKI (*Lebistes*); KRISTENSEN (*Zoarces*), and LEVER (*Serinus*)).

The new facts thus obtained were sufficient to justify an entire revision of the problem of the corpus luteum, and our investigations were at the same time extended to other components of the ovary, in order to discover what part the tissues or organs concerned play in the sexual-endocrine system.

In addition to *Rhodeus amarus* we examined the following:

#### SELACHII

*Galeus canis*  
*Acanthias vulgaris*  
*Mustelus laevis*  
*Scylliorhinus canicula*

#### TELEOSTII

*Lebistes reticulatus*  
*Xyphophorus helleri*  
*Zoarces viviparus*  
*Gasterosteus aculeatus*  
*Cyclopterus lumpus*  
*Lophius piscatorius*

#### AMPHIBIA

*Amblystoma mexicanum*  
*Megalobatrachus maximus*  
*Necturus maculatus*  
*Triton taeniatus*  
*Salamandra salamandra*  
*Bufo vulgaris*  
*Bufo calamita*  
*Rana temporaria*  
*Rana fusca*  
*Pipa americana*  
*Xenopus laevis*

#### REPTILIA

*Testudo graeca*  
*Seps chalcides*  
*Leiolepisma metallica*  
*Hemiergis decrescens*  
*Hemiergis peronii*  
*Mabuia striata*  
*Tropidonotus natrix*  
*Pelias berus*  
*Homalopsis buccata* . . .  
*Lachesis mutus*

#### AVES

*Passer domesticus*  
*Serinus canaria*  
*Gallus domesticus*

#### MAMMALIA

*Myotis myotis*  
*Erinaceus europaeus*  
*Talpa europaea*  
*Mus musculus*  
*Lepus caniculus*  
*Felis domestica*  
*Canis familiaris*  
*Ovis bovis*  
*Bos taurus*  
*Sus scrofa*  
*Homo sapiens*

The developing ovum and the follicle form, in all vertebrates, a topographical and functional whole. In all vertebrates the follicular wall consists of the same tissues—the granulosa and the theca. Only the placental mammals possess ova poor in yolk and with an antrum in their follicle (folliculus cavus), whilst all other vertebrates, together with a-placental mammals, possess ova rich in yolk, and a follicle without a cavity (folliculus solidus) (Fig. 69).

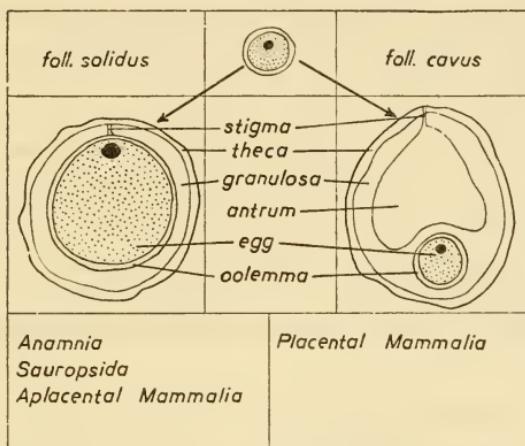


Fig. 69. Showing the difference between the folliculus solidus and the folliculus cavus.

The follicle has the double function of nourishing the growing egg and producing a glandular system which will serve for the subsequent care of the mature ovum. This ovo-follicular system is labile, so that the action of a single factor, generally from outside through stimuli from the anterior pituitary, causes profound modifications in the system. Its response to gonadotrophic pituitary stimuli is quite a different one from that of other glandular systems, whereas other responding organs such as the thyroid, the pancreas, the adrenal, etc. already exist in the form of completely developed functioning organs, being merely either excited, directed or checked by hormonal stimuli. It is quite different in the case of the glandular follicle of the ovary. Here, a gonadotrophic

stimulus meets only the basic tissues as elements from which afterwards the gland is to be developed and organized. In this process there is both disintegration and synthesis, the tissues having to be turned into hormone-forming cells by means of hyperplasia and hypertrophy. In fact, the corpora atretica and corpora lutea are transitory, rhythmically-created and only short-lived glands, whereas the other endocrine glands are formed during embryogenesis, and destined to last the whole of the animal's span of life. We must, therefore, attribute to the theca and granulosa a greater measure of autonomy in the form of inherent potentiality, and to the gonadotrophic stimuli a more active rôle than to the other endocrinial tissues and their hormonal stimuli, respectively. In the ovary these processes are on a higher plane, since here new organs are to come into being, whereas, in other endocrine glands, only the working rhythm is affected. In the ovary, in addition to exciting and directing the secretory function, the construction of the glands themselves is performed. Herein lies an essential difference between the two.

#### § 4. THE POTENTIALITIES OF THE FOLLICLE

The follicle of vertebrates possesses various potentialities, which may be actualized through the cooperation of different factors, and may lead to the formation of various follicular derivatives. The following factors are to be considered: (a) *the moment at which the change takes place*, whether before or after ovulation; (b) *the origin of the tissue*, whether it is the theca- or the granulosa tissue that is prominent; (c) *the rupturing* of the follicle; (d) *the dual anterior pituitary influence* on (i) the epithelium, leading to granulosa-luteinisation and (ii) the theca cells leading to theca-luteinisation; (e) the presence of a *critical period* for the tissues which are only able to respond at certain times.

Some part is further played by synergistic and antagonistic correlations among the component parts of the ovary, and between them and the pituitary, or between both of these and the adnexa. Added to this, there may be overlapping of

phases due to either anticipation or retardation of the degeneration, rupture, luteinisation or the critical period. Any disturbances in the fluid balance (cysts) and the blood supply (haemorrhage), and even disturbing influences of a nervous nature (shock) have an effect upon the ovo-follicular system. From the mutual competition of these factors there arises an astonishing multiplicity of formations such as is not to be observed in any other combination of tissues in vertebrates.

In order to obtain a clearer insight into this multiplicity we make a subdivision into the following four main groups:

- a. atresia;
- b. pre-ovulation corpus luteum;
- c. post-ovulation corpus luteum, and
- d. calyx.

All these follicular descendants, moreover, have a common plan of development (Fig. 70), consisting of the following 4 phases:—

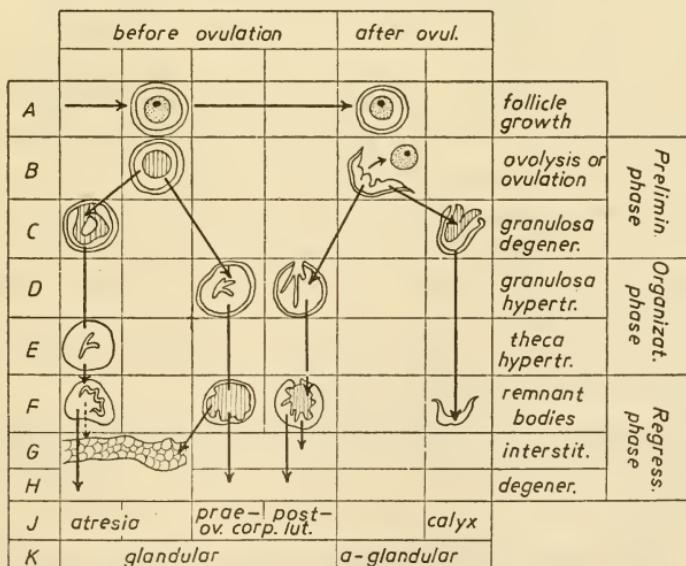


Fig. 70. Diagram showing the development of the follicular derivatives.

*a. Preliminary phase*, termed the  $\alpha$ -stage in our investigation of *Rhodeus amarus* (*vide p. 82*). We regard this as the introductory phase, which, in the pre-ovulation corpus luteum and atresia, consists of oolysis, and in the post-ovulation corpus luteum and the calyx, of ovulation. It can be shown in most cases that the ingestion is a response to a pituitary stimulus.

*$\beta$ . The "organization-phase"*; this is formed by the hyperplasia and re-arrangement of the tissue into a gland. It usually runs parallel with the  $\alpha$ -phase.

*$\gamma$ . The functional phase*, also called  $\beta$ -phase, represents the active phase of the gland, during which the hormone is secreted.

*$\delta$ . The regression phase*, also called  $\gamma$ - and  $\delta$ -phase, when the gland is removed after the cessation of its function. This brief process of creation, functioning and disintegration is characteristic of the internally secreting glands of the ovary.

*a. Atresia.* This is effected in the following manner:—

*a.* a thecatrophic pituitary factor sets up hypertrophy in the theca, producing luteinisation;

*$\beta$ .* an epitheliotrophic factor may either be absent or remain without effect owing to the granulosa cell being blocked up;

*$\gamma$ .* thecal substances (perhaps of an enzymatic nature) set up, either directly or via the granulosa, oolysis and granulosa degeneration. It frequently happens that the still intact granulosa prevents the luteinisation of the theca, whilst at the same time the degenerating granulosa fails to check luteinisation, which would seem to point to a correlation between these two tissues;

*$\delta$ .* the theca-lutein cells probably produce a sexual hormone which acts upon the adnexa;

*$\varepsilon$ .* the theca-lutein cells may persist as interstitial cells and in this state also produce a sexual hormone, probably the same (Fig. 71 A).

*b.* The *pre-ovulation corpus luteum* is formed in the following way:—

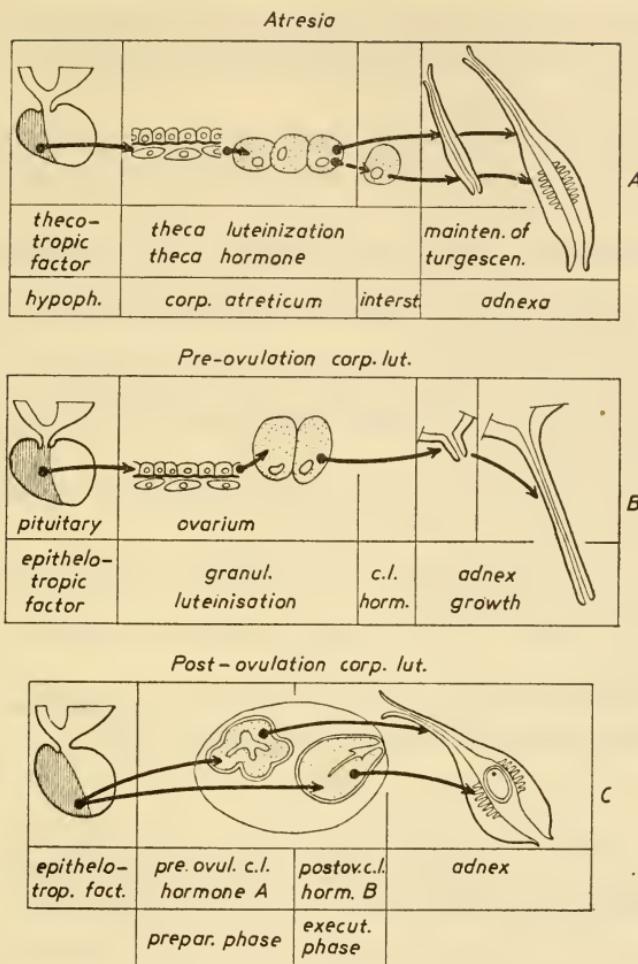


Fig. 71. Genesis and function of (A) the theca-lutein cells; (B) the pre-ovulation-, and (C) post-ovulation corpus luteum.

- a.* an anterior pituitary factor stimulates the granulosa, hypertrophy appearing as luteinisation;
- β.* a thecotrophic factor may either be absent or remain without effect on the blocked-up theca cell;
- γ.* granulosa substances (perhaps of an enzymatic nature) cause oolysis;
- δ.* the granulosa in most cases resorbs the degenerating ovum; only in exceptional cases (Elephantulus type) does this cell remain intact;

$\varepsilon$ . the granulosa-lutein cell produces a sexual hormone (oviductin in Anamnia; progesterone in Mammalia), which acts upon the adnexa;

$\zeta$ . only in exceptional cases do granulosa-lutein cells persist as interstitial cells; in most cases they succumb (Fig. 71 B).

c. The *post-ovulation corpus luteum* is formed as follows:

a. a rupture-factor (possibly supported nervously) causes rupture of the follicle;

$\beta$ . the ovum is carried away either fertilised or not;

$\gamma$ . a pituitary factor causes granulosa hypertrophy (granulosa luteinisation);

$\delta$ . a second pituitary factor may in some cases cause theca-hypertrophy (theca luteinisation);

$\varepsilon$ . the granulosa-lutein cell produces a sexual hormone acting upon the adnexa;

$\zeta$ . the theca-lutein cell also produces a hormone of its own, which acts upon the adnexa;

$\eta$ . the theca-lutein cells may persist as interstitial cells, whilst the granulosa cells succumb (Fig. 71 C).

The *calyx* is formed as follows:—

a. a rupture factor causes ovulation of the follicle;

$\beta$ . the ovum is carried away whether fertilised or not;

$\gamma$ . the pituitary cannot act on the granulosa cells;

$\delta$ . the pituitary may produce, in the most extreme case, a slight theca-hypertrophy;

$\varepsilon$ . the granulosa degenerates, possibly under the influence of thecal substances;

$\zeta$ . the theca turns into granulation tissue, and serves to close up the ovulation wound.

It will be seen from the above analysis that there is a distinct difference between thecal atresia and the granulosa formations (pre-ovulation corpus luteum), and that the three glandular follicular derivatives, corpus atreticum, pre- and post-ovulation corpus luteum, are the opposites of the non-glandular calyx-forms.

### § 5. SERIAL REPETITION OF THE POSSIBLE MODIFICATIONS IN THE FOLLICLE

In each of the four main groups referred to above we find analogous modifications, which are repeated and are founded upon the combinations of factors. In order to be able to keep these several follicular derivatives distinct from each other we have introduced the following terminology.

The different modifications within each of the four main groups are repeated serially because both the parts of the ovo-follicular system and the combinations of factors are after all limited, and each follicular derivative is the resultant of these two.

There are the following twelve possibilities of development:—

- a. the retention of the ovum or its extrusion;
- b. the gland may become either solid (solidus) or hollow (cavus);
- c. the gland may be formed from one kind of tissue only (simplex) or from a combination of theca- and granulosa-luteinisation (mixtus);
- d. the follicle may rupture (ruptus) or not;
- e. liquid may develop (cyst) or not;
- f. hyperaemia and haemorrhage (haemorrhagicus) may follow or not.

These possibilities have been summarized for all four groups together in the following table. It will be seen that out of 32 theoretical possibilities 28 have been realized in nature.

T A B L E III

	ret.	cavus	solidus	simplex	mixtus	ruptus	cyst	haemorrhagicus
ATR.	X	X	X	X	X	X	X	X
PRE.	X	X	X	X	O	X	X	X
POST	O	X	X	X	X	X	X	X
CAL.	X	O	O	X	X	X	X	X

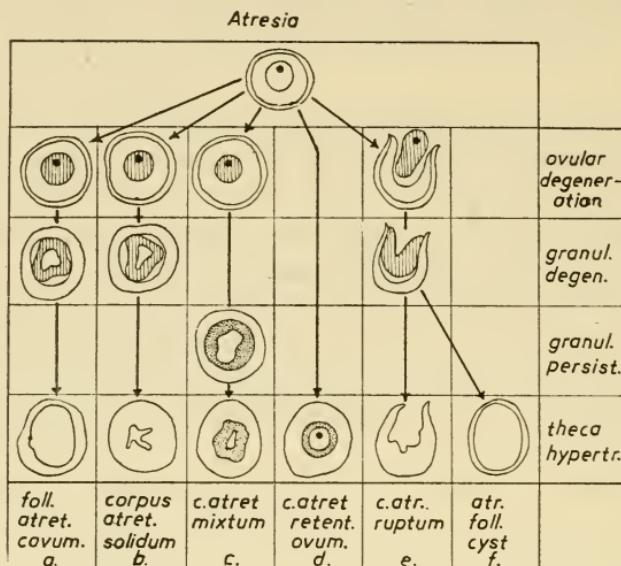


Fig. 72. Modifications of the atretic type.

a. *The modifications of the atretic type (Fig. 72).*

a. The *folliculus atreticus cavus* is characterized by degeneration of the ovum and of the granulosa, followed by only slight hyperplasia and hypertrophy of the theca, as a result of which a cavity remains. Several old follicles may, either in the normal course or after administration of certain gonadotrophic hormones, change into such atretic follicles (Fig. 72a).

β. The *corpus atreticum solidum* has the same preliminary phases; but in contradistinction to the atretic follicle there occurs marked hyperplasia and hypertrophy, owing to which the entire ovular and granulosa space not only becomes filled with theca-lutein tissue, but which also causes the whole gland to increase considerably in size (Fig. 72b).

$\gamma$ . The *corpus atreticum mixtum* shows only oolysis, and no degeneration of the granulosa, so that a mixture of these two follicular tissues is created. Although the persisting granulosa forms lutein cells, it remains small in amount, whereas the theca is hypertrophied and forms a large thecal gland.

Generally, indeed, both strata remain topographically separate from each other (Fig. 72c).

δ. The *corpus atreticum* with retention of the ovum is similar to the previous modifications, but goes one step further in that, apart from the persisting granulosa tissue, the egg cell, too, remains intact and usually does not pass to the degeneration stage until the regression phase has set in. A prominent feature in this, however, is again the strong augmentation and hypertrophy of the surrounding theca into theca-lutein gland (Fig. 72d).

ε. The *corpus atreticum ruptum* is distinguished from the preceding corpus atreticum by the occurrence of a rupture of the follicle accompanied by extrusion of the substance of the egg. The theca-lutein cells show a typically secreting glandular character (Fig. 72e).

ζ. The *atretic cyst*. By excessive liquor- or lymph-pressure the organization of the gland is checked, and a turgid cyst filled with liquid is formed; the corpus luteum cyst (Fig. 72f).

These are the possibilities of development in an atretic direction. In addition to either (a) incompletely or (b) completely constructed follicular derivatives consisting only of theca, we see the occurrence of other ones, consisting of a mixture of theca and granulosa (c, d). In addition to retention of the ovum (d) there is also a premature rupturing of the follicle (e).

b. *The modifications of the pre-ovulation type.*

With the pre-ovulation corpora lutea we class all those glandular descendants of the follicle in which the granulosa is hypertrophied into lutein cells before the follicle is ruptured, in which process the ovum usually succumbs. The atresia leads, owing to granulosa degeneration, to a theca gland, and the pre-ovulation corpus luteum to a granulosa gland. The destruction of the ovum is the only feature common to both. Again, the pre-ovulation type also shows an analogous modification-series (Fig. 73).

a. *The pre-ovulation corpus luteum cavum* is character-

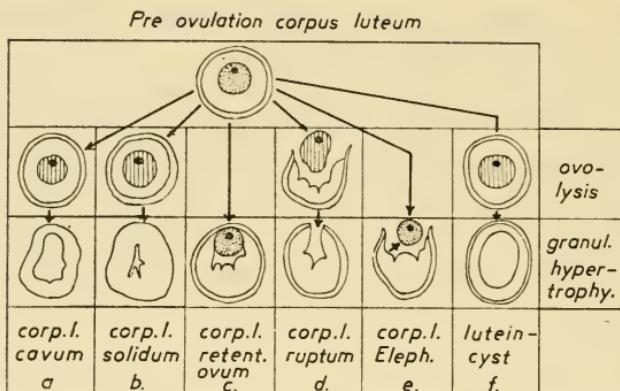


Fig. 73. Modifications of the pre-ovulation corpus luteum.

ized by an incomplete organization of the hypertrophying granulosa tissue into a gland, whereby only the wall thickens and a central cavity remains (Fig. 73a).

β. In the *corpus luteum solidum* the entire space is organized into one solid gland. In contradistinction of the *corpus atreticum*, this gland consists exclusively of granulosa-lutein cells and differs from the post-ovulation *corpus luteum* only in that the ovum disintegrates and that there is no rupture (Fig. 73b).

γ. *Pre-ovulation corpus luteum with retention of the ovum* is also a frequent occurrence. The granulosa tissue hypertrophies around the ovum and forms a solid lutein gland in which the egg cell is also destroyed, but not until the regression phase sets in. In mammals it usually occurs in corpora lutea which are of the same age as the post-ovulation corpora lutea in which there has been no ovulation. Such pre-ovulation corpora lutea, which have also been described by CORNER (1940) under the name of *corpora lutea aberrantia*, and by COLE, HOWELL, and HART (1931) as *corpora lutea accessoria*, have the same length of life as their sisters, the post-ovulation corpora lutea, and are subject to the same cyclic changes (Fig. 73c).

δ. The *pre-ovulation corpus luteum ruptum* is distinguished from the former by the oolysis and rupture occurring already during the preliminary phase, in which the substance

of the egg flows out. In this it resembles the corpus atreticum ruptum; it consists, however, chiefly of granulosa-lutein cells (Fig. 73d).

e. As transition to the post-ovulation corpus luteum we find a second form of the corpus luteum ruptum, in which the ovum is not destroyed, but emerges, at a far advanced stage of the corpus luteum, as a result of the rupture, and becomes fertilised. Since this particular modification has hitherto been observed as normal in only one mammal, i.e., in *Elephantulus myurus* (VAN DER HORST, 1940) we propose to call this corpus the "*Elephantulus type*". It forms the bridge to the actual post-ovulation corpus luteum, from which it is distinguished either by very early luteinisation or by very much retarded ovulation. As in many post-ovulation corpora lutea, it is not only the granulosa, but also the theca which luteinises (Fig. 73e).

ζ. Owing to an abundant liquor- and/or lymph pressure, during which the formation of glands is checked, a *corpus luteum cyst* (Fig. 73f) is formed.

c. *Modifications of the post-ovulation corpus luteum.*

a. The *post-ovulation corpus luteum cavum* owes its existence to the incomplete development of its granulosa gland after ovulation, a central cavity remaining free, while the corpus luteum remains small. It is identical with the corpus luteum menstruationis in women (Fig. 74a).

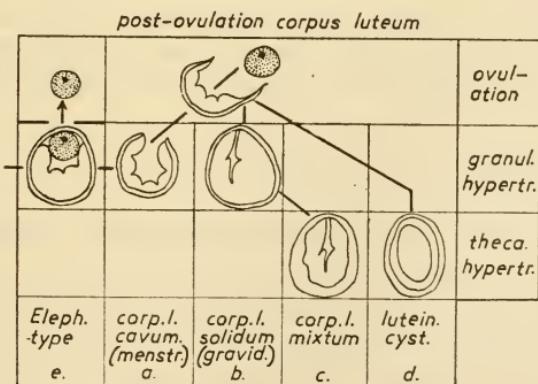


Fig. 74. Modifications of the post-ovulation corpus luteum.

$\beta$ . The *post-ovulation corpus luteum solidum* is characterized by the complete organization of the follicular cavity, after ovulation, into a solid gland: in mammals, the corpus luteum graviditatis. The hormone-secreting tissue consists, according to the species of animal, either exclusively of granulosa- or of both granulosa- and theca-lutein cells (Fig. 74b).

$\gamma$ . In the *post-ovulation corpus luteum mixtum* the theca also hypertrophies into lutein cells; during the organization of the gland this tissue mixes with the granulosa to form a functional whole (Fig. 74c).

$\delta$ . The *Elephantulus* type, being a transition between both corpus luteum types, may also be regarded as a *post-ovulation corpus luteum with temporary retention of the ovum*, rupture of the follicle occurring at a much later stage (Fig. 74e).

$\varepsilon$ . Finally, in this type, too, liquor- and lymph-pressure may lead to an incompletely organized *lutein cyst* after the ovulation-pore has closed up again (Fig. 74d).

Owing to the glandular character of these three groups, there is a well-developed system of bloodvessels and a certain degree of hyperaemia, caused also by pituitary agents. In many cases this may be so marked as to set up discharge of blood stamping the follicular derivative in question as haemorrhagic body. We know up to the present: the folliculus haemorrhagicus; the corpus atreticum haemorrhagicum and the pre- and post-ovulation corpus luteum haemorrhagicum, all of which, as so-called "bloodpoints" in the ovary, have engaged the attention of other writers (ASCHEIM-ZONDEK reaction).

#### *d. Modifications of the calyx type.*

The "calyx" denotes the ruptured ovum-less follicular wall showing no tendency to gland-formation.

$a$ . The *calyx simplex* represents the most normal form, in which, after rupture, the granulosa degenerates and is expelled, the remaining theca tissue closing up the ovary wall with granulation tissue (Fig. 75a).

β. The *calyx nutricius simplex* is a special form, adapted to the viviparity of certain fishes. In this case the calyx does not become involuted; the bloodvessels of the follicle form a capillary system through which nourishment (the "embryotrophe") is conveyed to the ovary-lumen. After the performance of this function this calyx also involutes into scar tissue (Fig. 75b).

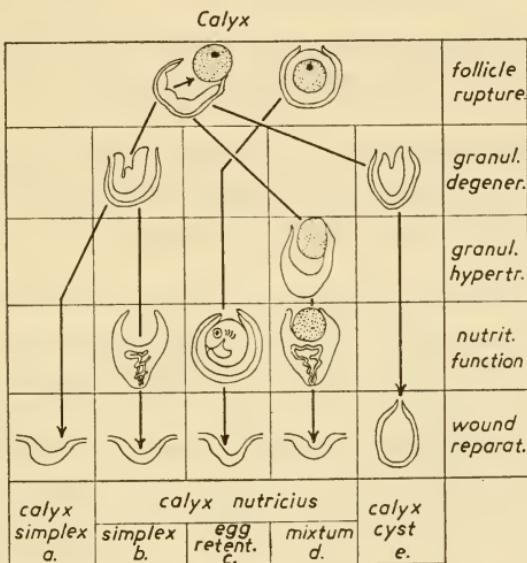


Fig. 75. Modifications of the calyx.

γ. The *calyx with retention of the ovum* is also an adaptation to the viviparity in fishes, and is at the same time a calyx nutricius; in this case, however, the ovum is fertilised and develops inside the ruptured follicle (Fig. 75c).

δ. The *calyx mixtus* belongs exceptionally to the same group of calyces nutriciae, differing from the first-named representative in that the more or less expelled granulosa hyperthrophies, and probably also has a certain secretory action. This constitutes as it were a first attempt, on the part of Anamnia, of forming a post-ovulation corpus luteum, and is, therefore, of particular phylogenetic significance (Fig. 75d).

ε. The *calyx cyst* may come into existence after the closure

of the rupture, through the pressure of lymph, and the auto-lysed, liquefied, granulosa tissue (Fig. 75e).

Thus we see that, in each of these groups, certain conditions repeat themselves, leading to analogous forms, although the origin and differentiation of the tissue and the moment of its formation show considerable mutual differences.

#### § 6. THE DERIVATIVES OF FOLLICULAR INVOLUTION AND THE INTERSTITIUM

The regressive phase of the various, often relatively large follicular derivatives may, according to the origin of their tissue and their function, or according to the species, adopt various forms.

a. The *corpus candidans*. In the case of the thecal atretic follicular derivatives a moment arises when the secretion of the lutein cells stops, and the connective tissue nature of the theca gets the upper hand again. In this, the thecal tissue frequently proliferates radially towards the centre. The originally thin hyaline membrane in the outer zone of the theca expands, forming thick folds. This is the membrane of SLAVYANSKI. Long after the other theca cells have degenerated and migrated it may still be seen in the stroma of the ovary (Fig. 70, F1).

β. The *corpus albicans*. As a rule the granulosa lutein cells are destroyed by degeneration, and are finally resorbed by leucocytes. The theca, however, which surrounds the corpus luteum either in the form of a layer of connective tissue or as theca-lutein cells, participates in the formation of glands, develops after its secretion has finished and proliferates through the granulosa tissue in course of dissolution. In this process, too, a membrane of SLAVYANSKI may be observed. Finally, this remnant shrinks and passes back into the stroma (Fig. 70, F3—F4).

6. The *interstitium*. The originally secreting tissue does not in all species die with the death of the gland. Remaining lutein cells may pass *en bloc* into the ovarian stroma.

Frequently they still show cyclic changes by growing smaller in the interestrus, expanding again in the estrus, and behaving as glandular tissue. In *Selachii*, BRETSCHNEIDER and VAN EGMOND observed a persistance of all granulosa-lutein cells of the pre-ovulation corpus luteum as large lamellae, constituting the major part of the large interstitium. The cells retain their glandular character and show cyclic changes as long as the gland is well supplied with blood.

To summarize, we may say that there exists a morphological, and probably also functional, contrast between corpora atretica and corpora lutea. The atresia produces a theca gland; the corpus luteum forms a granulosa gland. In so far as mixed forms occur, theca-luteinisation predominates in the corpus atreticum, and granulosa-luteinisation in the corpus luteum, both as regards the tissue and as regards hormone production. The corpus atreticum mixtum, moreover, occurs only rarely; if at all, only in young follicles. The pre- and post-ovulation corpora lutea, on the contrary, show, morphologically, an almost continuous series, so that in borderline cases, such as the pre-ovulation corpus luteum with retention of the ovum, or the *Elephantulus* type, we may frequently be in doubt as to the group with which it should be classed.

A different question altogether is which hormone is formed by the pre-ovulation corpus luteum, and which by the post-ovulation corpus luteum. For an answer to this question, adequate data are lacking in many cases. We know that the pre-ovulation corpus luteum in *Rhodeus* and *Lophius* forms *oviductin*, and that the post-ovulation corpus luteum in mammals produces *progesterone*. These two sexual hormones are not identical, as was already stated in the Introduction. The products of the other pre- and post-ovulation corpora lutea, of amphibia, reptiles and birds, or of the atretic follicular derivatives, are unknown. Oviductin and progesterone represent, after all, the two extremes; and the question of where, in the range of vertebrates, oviductin stops and progesterone begins is of some theoretical, and, maybe, also of practical importance.

## § 7. THE SIGNIFICANCE OF THE CORPORA LUTEA AND OTHER FOLLICULAR DERIVATIVES IN THE SEXUAL-ENDOCRINE ORGANIZATION OF VERTEBRATES

Apart from morphological data relating to the development of follicular derivatives we are chiefly concerned here with their significance within the sexual-endocrine organization of the particular species with which we are dealing. With the exception of what we already know concerning the post-ovulation corpus luteum in mammals, however, we are unfortunately still at the beginning of our studies, and we can only guess at the real extent of their importance. Only by future experiments will it be possible to obtain confirmation of such hypotheses or their replacement by more correct views.

a. *The atretic follicular derivatives* probably produce a sexual hormone whose function is to maintain the adnexa of the gonad, during periods of little sexual activity, and to bring these tissues into such a condition that a response may be made when the moment of heightened activity has come. The preparation of the adnexa in puberty is an example of this. It is accompanied by a most intensive formation of atretic follicular derivatives. These atretic phenomena are chiefly limited to birds and mammals (Fig. 76A).

β. *The significance of the pre-ovulation corpus luteum.* BRETSCHNEIDER and DUYVENÉ DE WIT (1937-'39-'40) have shown that, in the bitterling, the hormone of the pre-ovulatio corpora lutea is responsible for the growth of the oviduct. They have called this hormone "oviductin", and have succeeded in preparing it from the ovary of another fish (*Lophius*). With this it was proved that in the ovary of fishes, after stimulation from the hypophysis, a sexual hormone is formed which acts upon the oviduct, and that this hormone is produced in follicular derivatives formerly regarded as degenerative formations. With this the bridge was laid between Anamnia and Mammalia, and a similarity to the sexual-endocrine organization of vertebrates demonstrated. In both fishes and amphibia the pre-ovulation corpus

luteum is the only secretory gland in the ovary, and solely responsible for any changes in the genital apparatus (Fig. 76B).

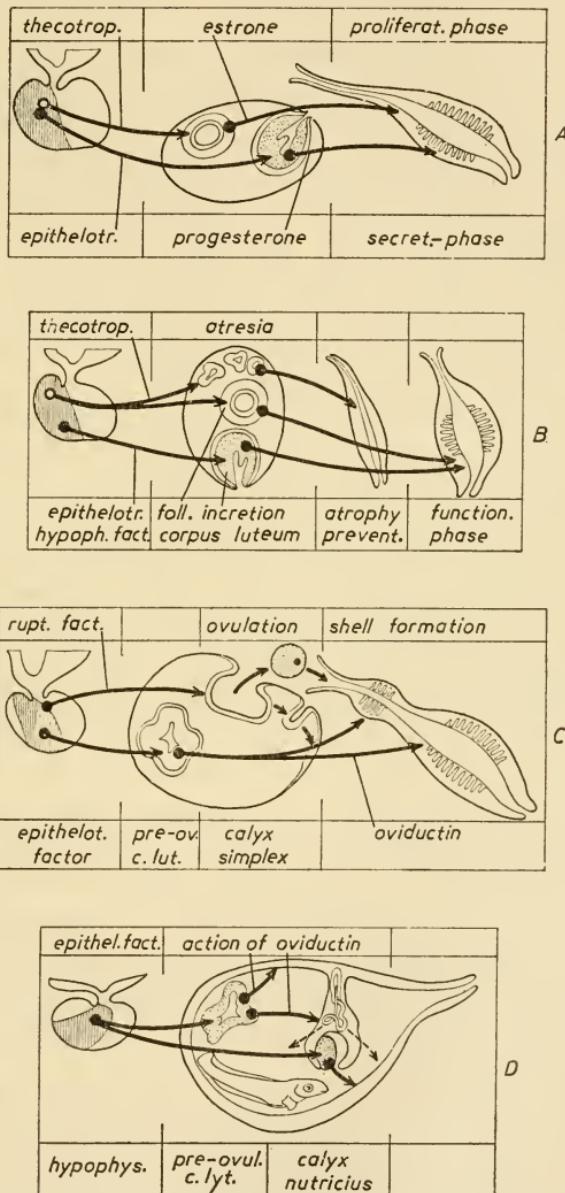


Fig. 76. The significance of the corpora lutea and other follicular derivatives in the sexual-endocrine organization in vertebrates.

*γ. The significance of the post-ovulation corpus luteum.* The sexual-endocrine organization of mammals has hitherto been the centre of interest, and is accordingly most widely known.

We now know that, probably through a pituitary factor acting on the theca, the latter secretes, in the follicular stage, the estrogen which causes the uterus to hypertrophy and prepares it for pregnancy (the so-called proliferation stage) (Fig. 76C). After ovulation, the granulosa luteinises under the influence of a second pituitary factor, and produces progesterone, which brings the uterus, primed by estrone, into the secretion-phase, thus rendering pregnancy possible. The post-ovulation corpus luteum persists and continues to function for a long time during pregnancy.

Cooperation with the corpora atretica should be viewed in this way, that it guards the adnexa against involution, both before puberty and during the interestrus, so that with the appearance of the first corpus luteum a normal response may be assured again (Fig. 76B). This applies chiefly to mammals. In birds and reptiles, however, post- and pre-ovulation corpora lutea appear both simultaneously and in succession, (and frequently in equal numbers). From the frequency with which the pre-ovulation corpora lutea occur in the interestrus and in youth we may draw the conclusion that here, too, they play their part in the preparation of the oviduct, for shell-formation in oviparous reptiles and birds, whereas the post-ovulation corpus luteum in viviparous reptiles flourishes for a longer period when it serves for the nourishment of the developing ovum. In oviparous *Sauropsidans* the post-ovulation corpus luteum is only present for a brief period; gland formation is incomplete, and its function in providing the ovum with its shell probably of minor significance (Fig. 76C).

*δ. The significance of the calyx.* Ovulation leaves a scar in the ovary wall. It is the task of the calyx to repair this defect. The calyx is typical of *Anamnia*, and is always found in conjunction with the pre-ovulation corpus luteum (Fig. 76C), which appears already long before ovulation, keeps the

adnexa up to a certain level of development (ovipositor in *Rhodeus*; shell-gland in *Amphibia* and *Selachii*), so that, after the completion of ovulation, all that remains for the calyx is to close up the ovulation porus. A different significance is allotted to the ruptured follicle in all viviparous fishes, where the further care of the ovum is of importance. In this case the calyx does not involute, but rearranges itself, through strong vascularisation, for a nutritive function, and does not serve in closing up the wound until after the birth of the foetus. Since pregnancy, in these viviparous fishes, takes place in the ovary, the changes necessary to it extend over the entire organ. Strong hypertrophy and extensive vascularisation are characteristics of the pregnant ovary. Here, again, numerous pre-ovulation corpora lutea appear in the pro-estrus and during pregnancy. Their importance probably lies in the production of hypertrophy, hyperaemia and vascularisation of the ovary, and in the calyx nutricius (Fig. 76D). Owing to the long persistence of these calyces nutricii (in *Zoarces* up to 4 months), the granulosa is, in many cases, luteinised. In this way the calyx nutricius mixtus in *Zoarces* is formed, which, by its granulosa-luteinisation, shows a similarity to the post-ovulation corpus luteum, and is, therefore, of theoretical significance in the corpus luteum problem.

#### § 8. THE FREQUENCY OF THE FOLLICULAR DERIVATIVES IN THE DIFFERENT GROUPS OF VERTEBRATES

In order to obtain an insight into the manner in which the numerous follicular derivatives here described are distributed over the various groups of vertebrates, we have examined more closely representatives from all the groups. In Table III these data have been summarized, together with some additional facts from the literature. We see that, in *Anamnia*, the pre-ovulation corpus luteum is associated with the sexual-endocrine organization, and that the ovulated follicle, as calyx simplex, is represented only by a scar. An exception to this—as we stated before—is formed by viviparous

fishes (e.g., *Zoarces*), whose calyx nutricius may show a weakly luteinised granulosa.

In *reptiles* we see for the first time, phylogenetically, a genuine post-ovulation corpus luteum. We can distinguish two groups: (a) the oviparous reptiles, with a short-lived, incompletely organized post-ovulation corpus luteum, and (b) the ovoviviparous and viviparous reptiles, with a long-lived, completely organized post-ovulation corpus luteum.

In *birds* there is added to this double provision of corpora lutea, as an inheritance from their Sauropsidan-organization, the atretic element. As in oviparous reptiles, the post-ovulation corpus luteum shows only incomplete organization. At the same time there is abundant formation of corpora atretica, which, in the case of follicles with large ova rich in yolk, frequently become ruptured, and give rise to a large corpus atreticum ruptus.

In *Monotremata* there is no pre-ovulation corpus luteum, but a well-formed post-ovulation corpus luteum, accompanied by numerous large corpora atretica. There are two phenomena which characterize *Monotremata*: (a) numerous large corpora atretica *rupta*—probably a result of oviparity and the fact that their ova are rich in yolk as in *Sauropsidans*, and (b) the well-formed post-ovulation corpus luteum for the provision of nourishment for the young, pointing to the condition in placental mammals.

In most *placental mammals* the post-ovulation corpus luteum predominates; it is, however, always accompanied by different forms of atretic follicular derivatives. During the last decade some exceptions of more theoretical importance have come to light; for example, the *Elephantulus* type, in which ovulation is preceded by the organization of the corpus luteum, and its hormonal function. Closely akin to this form of retention of the ovum is a second form of retention of the pre-ovulation corpus luteum, which occurs regularly in addition to the ordinary post-ovulation corpus luteum in the rat (LONG and MCLEAN, 1922); the mare (COLE, HOWELL and HART 1931); the porcupine (*Erethizon dorsatus*, MOSSMAN, 1940) and *Macaca thesus* (CORNER,

1936-'40). In man, a pre-ovulation corpus luteum (LEOPOLD, 1883), and a ruptured and ill-developed follicle which might, morphologically, be regarded as calyx (DUBREUIL, 1940), have also been described as exceptional cases (*vide* Table IV).

TABLE IV

Frequency of follicular derivatives

group	term.	calyx and pre-ovul. corp. lut.	pre-ovulat. post-atr.c.l.	atretic and post-ovul. corp. luteum	remaining mammals
	ruptum				
corp.	retent. ovum				
atretic	mixtum				
	simplex				
post-ov.	solidum				
c.l.	cavum	○			
prae-	<i>Elephantulus</i> typ.				
ovul.	ruptum				
c.l.	retent. ovum				
	simplex				
calyx	nutr. ret. ovum				○
	nutr. simplex				homo
	simplex				horse, rat, <i>Macacus</i>
					<i>Elephantulus</i>
					<i>Monotremata</i>
					<i>Aves</i>
					<i>vivip.</i> } <i>Reptiles</i>
					<i>Amphibia</i>
					<i>Teleost.</i>
					<i>Selachii</i>
					<i>Cyclostomata</i>
					Anamnia
					Amniota
					remaining mammals

These exceptions confirm our view that the follicle in vertebrates has great potentialities.

TABLE V

atres.									
post-ov. corp. l.									
prae-ov. corp. l.									
calyx									
		Cyclost.	Selach.	Teleost.	Amph.	Reptil.	Aves	Monot.	
		Anamnia				Amniota			
						Sauropsida		Mammalia	

## § 9. SUMMARY

To summarize (Table V), we cannot draw a sharp line of distinction either between Anamnia and Amniotes or between Sauropsidans and mammals, since the various follicular derivatives overlap phylogenetically. The pre-ovulation corpus luteum occurs both in Amphibia and in mammals; the atretic follicular derivatives are found in birds and also in reptiles; and the corpus atreticum ruptus is typical of both birds and Monotremata. We may affirm, therefore, that the significance of the calyx and the pre-ovulation corpus luteum from the Cyclostomata to the mammals diminishes in the same proportion as the significance of the post-ovulation corpus luteum and the atresia increases. Where, in this sequence, secretion of oviductin finishes and that of progesterone begins has yet to be investigated.

## IV. THE CYCLIC CHANGES IN THE SEXUAL- ENDOCRINE ORGANIZATION OF NON- MAMMALIAN VERTEBRATES

The aim of our investigations in the sexual-endocrine field was to elucidate the sexual cycle. We first studied the sexual cycle of the *female bitterling* (BRETSCHNEIDER, DUYVENÉ DE WIT, GOEDEWAAGEN, VAN IERSEL, and MELTZER), in which growth of the ovipositor clearly indicates the internal condition of the sexual-endocrine system. Following this, we studied the sexual cycle of other non-mammalian vertebrates, e.g., that of two viviparous fishes, *Lebistes reticulatus* (BRETSCHNEIDER, JASKI) and *Zoarces viviparus* (KRISTENSEN, BRETSCHNEIDER), and that of *Bufo bufo* (BRETSCHNEIDER). Our investigations into the sexual cycle of *Tropidonotus natrix* and *Seps chalcides* (BRETSCHNEIDER), and *Serinus canaria* (LEVER), are not yet completed.

### § 1. THE SEXUAL CYCLE OF RHODEUS AMARUS ♀

By a sexual cycle we mean the succession of changes occurring in the sexual apparatus, in estrus and in anestrus. Since *Rhodeus* belongs to the monestrous type, the cycle extends over an entire year. We have taken the beginning of anestrus as the starting point of our description of the cycle, since, at this time, sexual activity is at its lowest level.

Estrus lasts about 2 months (May and June, or, if Spring is early, estrus may begin about the middle of April); and, as described above on p. 70, it is rhythmical. Anestrus lasts 8 months (July to February); and pro-estrus, or the preparatory phase, two months (March and April). We have examined the following stages in the complicated cyclic process: (a) ovogenesis; (b) corpus luteum formation; (c) the pituitary regulation of both, and (d) growth of the

ovipositor. In this way, coordination between (1) the hypophysis, (2) the gonad, and (3) the ovipositor became apparent.

a. *Inter-estrus.* The central point of sexual life is the maturation of the ovum. Development of the ovum and formation of the corpus luteum have already been described cytologically (p. 77). We shall now, therefore, deal mainly with statistical data concerning the ovary. In the month of July following the last oviposition, maturing and mature oocytes (1100—2000  $\mu$ ) are absent from the ovary, having been discharged. Apart from a small number of medium-sized oocytes (200—500  $\mu$ ), the ovary now contains a very large number of small oocytes (10—200  $\mu$ ), as seen in the ovary of a young animal (Fig. 77A). This condition lasts only a short time, for in the same month medium-sized ova develop from this reservoir of small oocytes, and they in turn give rise to larger ones. It is probable that this growth of gametes is initiated by the hypophysis. During the autumn and winter the formation of medium-sized ova from smaller ones proceeds slowly, while the number of large ova remains at the same level. These large cells, however, also grow slightly during this time, i.e., from 500 to 800  $\mu$  (Fig. 77A).

b. *Pro-estrus.* By the end of the winter (February) the ovary possesses a few small oocytes, and a large number of medium-sized ones. During the next 2 months of pro-estrus the number of medium-sized oocytes diminishes rapidly with the formation of pre-ovulation corpora lutea (Fig. 77B). Even large-sized oocytes participate in this change. The abundant pro-estrous formation of corpora lutea, however, is not accompanied by an equally intensive growth of the ovipositor, as might be expected (Fig. 77C). From February to April the ovipositor shows only slight growth, during which the tissue gradually begins to respond to successive hormonal stimuli, until, during estrus, the normal degree of sensitivity is reached. The corpora lutea in pro-estrus, therefore, it is suggested, sensitise the ovipositor. A further characteristic of this period is an intensive growth of the large oocytes into maturing ones of 2000  $\mu$ .

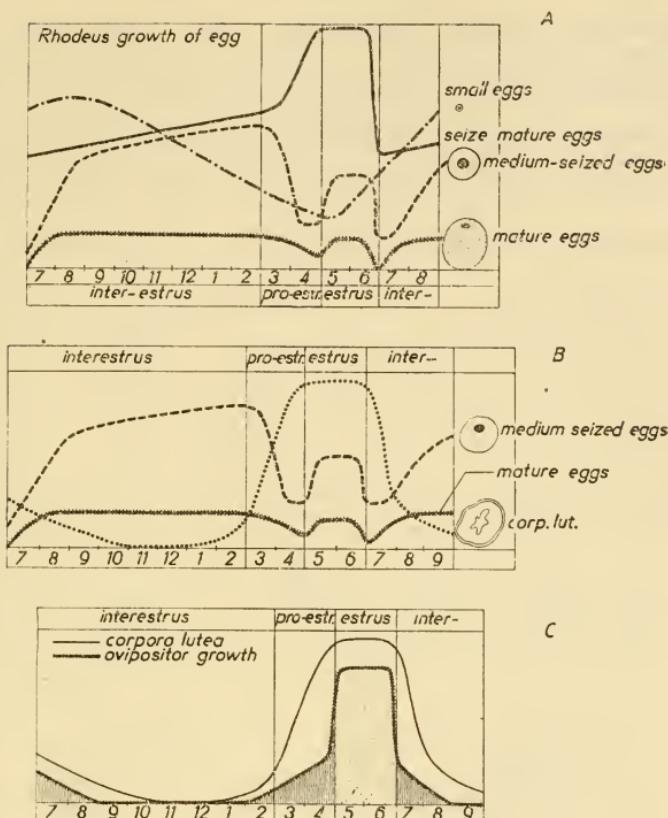


Fig. 77. Relation between the growth of the eggs, the formation of corpora lutea and ovipositor growth during the cycle in *Rhodeus amarus*.

c. *Estrus*. At the end of April or the beginning of May the first ova are deposited, and after this at intervals until the end of June. From investigations by MELTZER the following facts have come to our knowledge. As we have already remarked, development of the ovipositor is complete only when both male bitterlings and mussels are available. It has been proved that the male bitterling secretes a substance into the water which causes the ovipositor to grow (GOEDEWAAGEN - unpublished). JASKI called the substance, in the case of *Lebistes*, "copulin"; but without the mussel, growth is incomplete and oviposition never takes place. When, during spawning time, the ovipositors of female bitterlings kept in

an aquarium together with both males and mussels are measured regularly (for the method of measurement *vide* p. 4), we find that the changes in length (Fig. 78) are cyclic. The cycle differs with each female. Oviposition coincides with the top of a cyclic curve. In some females, during

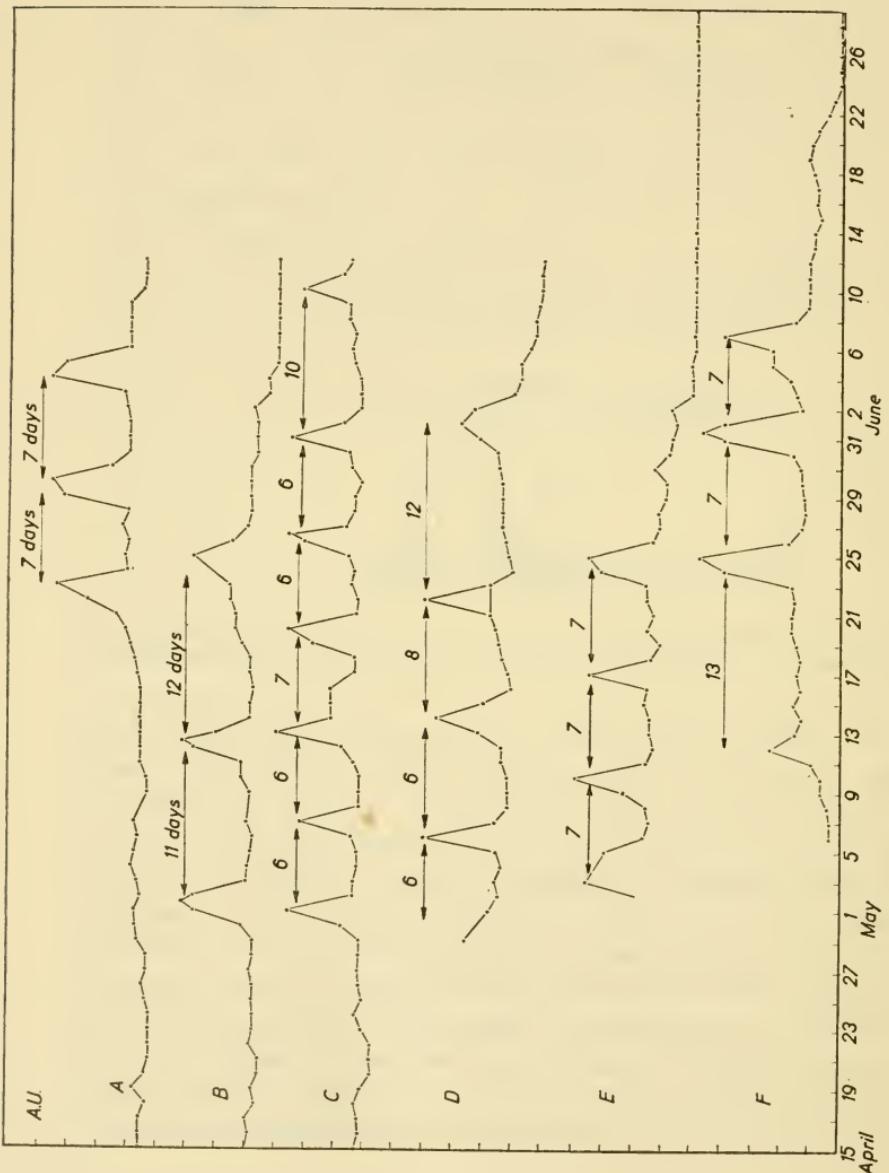


Fig. 78. Periodical fluctuations in ovipositor growth in the female beetle during spawning time. Oviposition takes place at each of the peaks.

the entire time of spawning, oviposition occurs only twice; in most cases it occurs 4 to 5 or even 7 times. It appears from the curves that the ovipositor grows periodically, in periods of, generally, 6—8 days; sometimes, however, there may be periods of from 11—13 days. It is possible that, in these fishes, an ovulation-phase is missed. It is further remarkable that the growth of the ovipositor takes place in two stages. The lengthening from 0—5 A.U. may take weeks; the lengthening from 5 A.U. to the complete length occurs very quickly — usually within 24 hours. Shortening after oviposition also takes place rapidly, the starting length of about 5 A.U. generally being reached again within 24 hours. During the spawning period, however, the length of the ovipositor is never reduced to 0 A.U. During this period it fluctuates between 5 and 8 A.U., reaching a top oviposition occurring at intervals of about a week.

When we analyse the different stages of ovipositor growth during spawning time we distinguish the following (Fig. 79):—

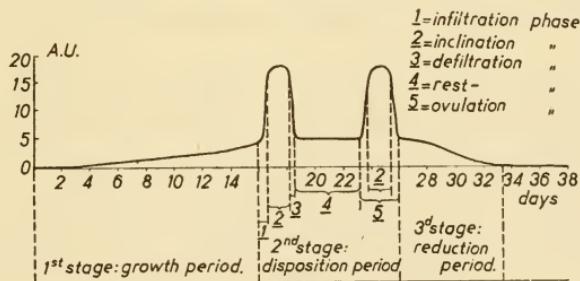


Fig. 79. Analysis of the different stages of ovipositor growth during spawning time.

*1st stage.* Length 0—5 A.U. The ovipositor is in the growing period. Histologically, this phase is characterized, at any rate under experimental conditions, by numerous mitoses.

*2nd stage.* Length 5—24 A.U. The ovipositor is now at the laying stage. It is periodically able to attain rapidly a maximum-length, rendering egg deposition possible. The following phases may be distinguished:—

- a. *Rest-phase*; length, 5—8 A.U.
- b. *Ovulation phase*; subdivided into:
  - a. *Infiltration phase*. The ovipositor lengthens through infiltration with lymph and expansion of the tissue-collagen to a length of about 16 A.U.
  - b. *Inclination phase*. During this phase ovulation takes place. The ovipositor may lengthen still further, but only by 3 A.U. at most, reaching a final length of between 16 and 24 A.U. A characteristic of this phase is the oblique position to the female: head down, tail up; hence the term "inclination-phase".
  - c. *Reduction phase*. The ovipositor is reduced to the same length as during the rest phase.

*3rd stage*. Reduction phase. After spawning time the ovipositor tissue atrophies.

The scheme mentioned above is further clarified by Fig. 80. Outside spawning time the uro-genital papilla is visible, in the form of a small elevation between the anus and the anal fin. At this stage, growth of the ovipositor proceeds extremely slowly. The laying period coincides with the possibilities of oviposition. The infiltration phase often lasts 12 hours, but may extend to 24 hours..

Within 24 hours, therefore, the greatest lengthening of the ovipositor takes place. As a rule the inclination phase continues for 24

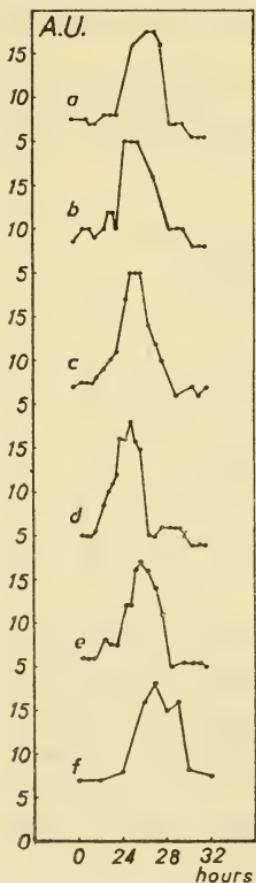


Fig. 80. Curves of carefully measured ovipositor growth during the ovulation period in several female bitterlings.

hours. The ova are deposited in the mussel one at a time, at intervals varying from one minute to one hour or even longer. There finally follows the reduction phase, which may last between 12 and 24 hours. The entire ovulation phase generally lasts about 48 hours.

We should here insert a description of the *mechanism of erection of the ovipositor*. The ovipositor is placed in a rigid state into the exhalant siphon of the freshwater mussel. The ovipositor itself is completely flaccid, and cannot be pushed into the mussel in that state. When a mature ovum arrives at the base of the ovipositor it is pushed into the ovipositor by contraction of the muscles of the urinary bladder. The urine cannot run away through the ovipositor, because the ovum acts as a plug. When the ovum is pushed towards the tip of the ovipositor, that part of the ovipositor behind it is entirely filled with urine, which causes a certain rigidity. In this rigid state the ovipositor is introduced into the mussel, and is able to penetrate deeply enough into the mollusc for the safe deposit of the ovum. As soon as the ovum has been forced out of the ovipositor the liquid under pressure flows away and the ovipositor relaxes completely.

This course of events was observed in ovulations that had failed, and in which the ova had missed the mussel. In normal cases the ovum glides through the ovipositor so quickly that the tube is rigid for only a very short time and it is difficult to follow the process closely, especially as the ovipositor is for the greater part inside the shells of the mussel (*vide* Fig. 81 and 82).

An interesting detail is the behaviour of the male in display and during oviposition. In estrus the male is very finely coloured. On the breast and belly the colours become a yellowish orange to a warm red, while the back- and anal fins grow a deep red and are marked with conspicuous edges. Another striking feature is the steel-blue, irradiating side-line. On the nose, on either side of the nostrils, small chalky-white warts appear, the so-called pearl-form organs, consisting of epithelial cells closely crowded together. When the male displays in spawning time it is inclined to place itself near a mussel. The latter is regarded as the centre of the male's territory, and from here fishes venturing inside this domain are

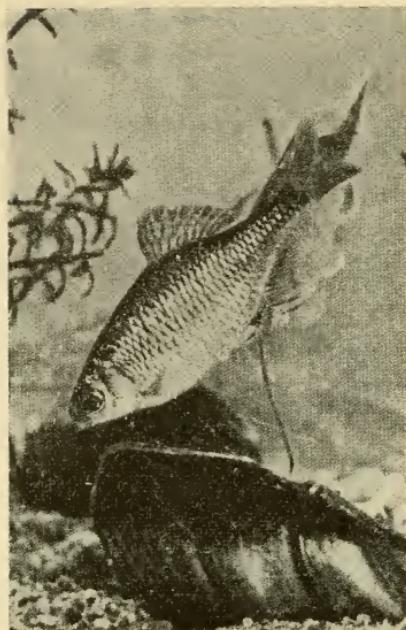


Fig. 81.

Bitterling pair near a mussel.

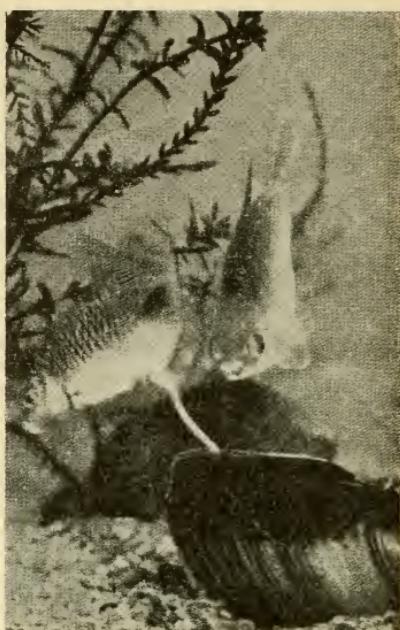


Fig. 82.

Female bitterling in oviposition.

continually chased away. Not only are males of the same species driven off, but all sorts of other fishes as well, even females with long ovipositors. Those female bitterlings, however, which, apart from possessing a long ovipositor, also adopt a certain posture, namely with the head inclining slightly downwards (inclination-position) appear to attract the special attention of the male. These females do not take flight as the male rushes at them impetuously, but rather convey the impression of being apathetic. When a male comes in the neighbourhood of such a female its behaviour suddenly changes. Displaying himself, and trembling all over, he slowly swims before the female in the direction of the mussel. The female seems fascinated by these tremblings, and follows slowly until it comes near to the mussel. There it inspects the filaments of the exhalant siphon, and in the most favourable case places itself with its tail above the siphon of the mussel, its head slightly downwards, and with a sudden forward movement shoots its ovipositor into the opening. During this manoeuvre, which lasts only a fraction of a second, the displaying male performs extremely rapid trembling movements, which probably constitute the actual ovulation stimulus to the female. As soon as the female has deposited her egg and swims away the male, shining in brilliant colouring, discharges his sperm from above into the same opening.

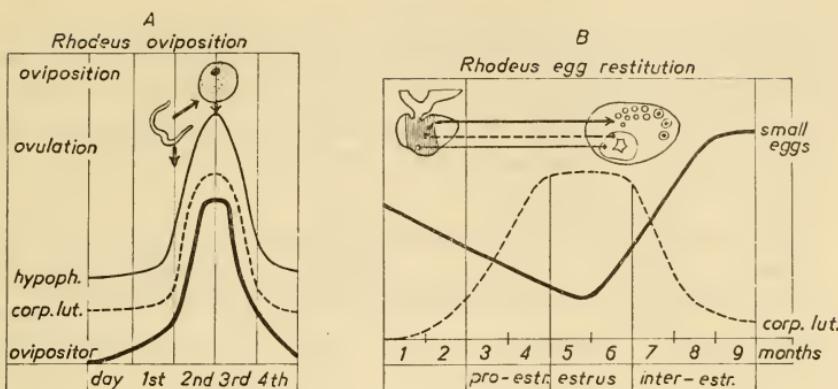


Fig. 83. Relation between the rhythm of ovipositor growth, the formation of corpora lutea and the hypophysis in the female bitterling.

The rhythm in the growth of the ovipositor has its concomitant in a similar rhythm in the ovary and in the hypophysis. The curves in Fig. 83A during estrus only show the maxima; in reality they run up and down. In these months we find, in addition to animals with numerous corpora lutea, others without any corpora lutea, and the length of the ovipositor varies correspondingly. In accordance with this, VAN IERSEL (*vide* p. 70) found, during this time, in addition to hypophyses with intensive basophilia throughout the whole of the lobus anterior, others possessing only small basophil islands, such as we find in anestrus.

A day before oviposition the follicles ovulate; we then find calyces in the wall of the ovary, and lying loose in front of the mouth of the oviduct. Oviposition lasts only one day, after which there is a pause of from 6 to 8 days before the next oviposition. Even before the gonad is deprived of its stock of ova owing to oviposition and copious production of corpora lutea, and already during estrus, restitution of ova commences. The curve of the small oocytes reaches its lowest point in May, rising rapidly directly after. Our experiments with hormones described on p. 52 and 53, in which there was no oviposition, but considerable production and consumption of corpora lutea, also show the same

rapid restitution of ova after repeated growth of the ovipositor. We have therefore formed the opinion that the oviductin produced stimulates the hypophysis into emitting a gonadotropic factor, which, in the gonad, causes development of young oocytes, thus maintaining the appropriate number of gametes (Fig. 83 B).

We see, therefore, that in the course of the sexual cycle of *Rhodeus amarus*, the ovary, as producer of gametes and as hormonal gland, is linked with the hypophysis on the one hand and the ovipositor on the other. In this, coordination of the phenomena of estrus is characterized by a certain rhythm in each of the 3 organs concerned.

## § 2. THE SEXUAL CYCLE OF ZOARCES VIVIPARUS

There are few fishes that possess such a clearly defined indicator of the changes in their sexual-endocrine organization as the ovipositor in *Rhodeus*. In most fishes oviposition as such takes place in a very simple manner, and accordingly we find only limited preparatory forms such as pre-ovulation corpora lutea, and expansion of the oviduct. In viviparous fishes, on the other hand, the care of the developing ovum requires a number of accessory organs and numerous changes. For this reason BRETSCHNEIDER and KRISTENSEN have investigated the sexual cycle of *Zoarces viviparus*.

*Zoarces* is one of the "ovary-breeders"; the ova are fertilised in the ovary and there develop into young. Pregnancy lasts about 4 months, and may take place in one animal from April to July (pregnancy a), and in another from September to January (pregnancy b). *Zoarces* from the same locality (North Sea, near Den Helder, North Holland) proved to be subject to both pregnancies, although pregnancy occurred with greater frequency in the autumn.

It is improbable that a fish has two pregnancies in one year. Since the animal material in our experiments appeared to be mixed, the curves show both pregnancies within the span of one year. We accordingly find, both in April and in August and September, mature oocytes (Fig. 84), formed

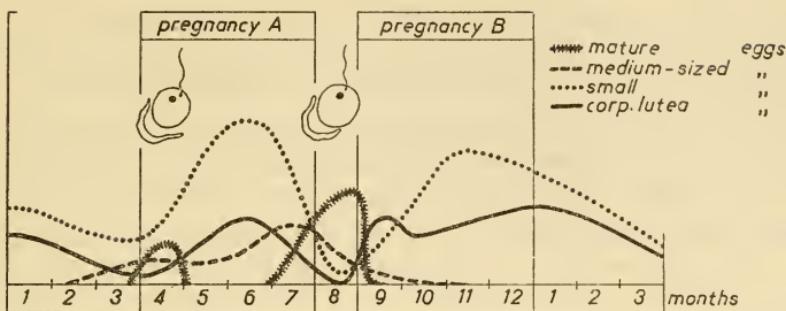


Fig. 84. Showing the relation between egg-development and corpus luteum formation in *Zoarces viviparus*.

from medium-sized ova, which in their turn were formed from smaller ones.

As in *Rhodeus*, the growth of the ova before ovulation is completed in the space of 2 months at an accelerating speed (Fig. 84). While the mature follicles are ovulating, the medium-sized oocytes develop into pre-ovulation corpora lutea, which remain in evidence during the whole of pregnancy, and finally evolve, after passing through the ingestion-, functional- and regression-phases. Probably two generations of corpora lutea make their appearance during each pregnancy (Fig. 85). The fertilised oocytes of 20 mg

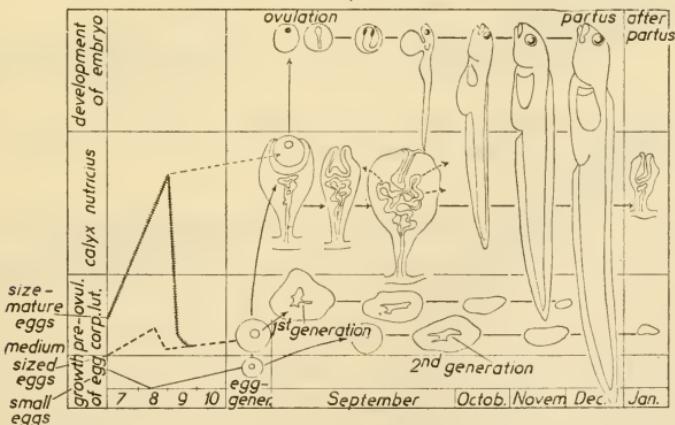


Fig. 85. Showing the relation between egg-development and embryonal development in *Zoarces viviparus*.

grow in 4 months into young fishes 240 mg in weight and 44 mm long. This intensive increase in weight is made possible by the copious secretion of embryotrophe by the ovulated follicles, the calyces nutriciae (*vide* p. 106). In these calyces the bloodvessels develop strongly, while the cavities become filled with lymph. They do not involute until some time after parturition.

As was described on p. 107, the granulosa does not usually degenerate in this long-lived calyx, but changes into lutein tissue, so that, in this case, we may already speak, theoretically, of a post-ovulation corpus luteum. From the frequency of these luteinised calyces, and the simultaneous presence of pre-ovulation corpora lutea we conclude that both are subject to the same hypophysial influences. The times of their respective stages of development also correspond: in December, the functional phase ( $\beta$ -stage); in January, the regression-phase ( $\gamma$ ) (*vide* Fig. 86). In view of its association with

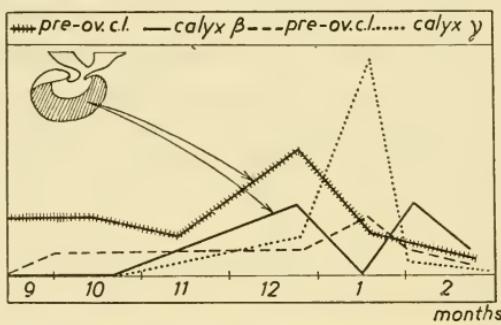


Fig. 86. The correlation between the number of pre-ovulation corpora lutea and  $\beta$ -calyces during pregnancy in *Zoarces viviparus*.

pregnancy, we regard oviductin as having a stimulating effect on the ovary wall and on the calyces nutriciae, causing hypertrophy and marked hyperaemia of the tissues. These phenomena are seen in *Rhodeus* during the growth of the ovipositor. In *Zoarces*, after considerable loss of ova and the formation of corpora lutea (April or September), there follows a rapid restitution of ova (Fig. 86), probably as a result of a strong hypophysial stimulus.

To summarize we may say that in this viviparous fish the sexual cycle is regulated by (a) co-ordinated ovular growth, followed by internal fertilisation, and care of the foetuses with the aid of an embryotrophe secreted by the calyx nutritius, and (b) intensive corpus luteum formation (pre-ovulation corpora lutea), which is often supported by luteinisation of the calyx granulosa. It is probable that sexual-activity may occur in spring as well as in autumn.

### § 3. THE SEXUAL CYCLE OF *LEBISTES RETICULATUS*

JASKI (1938) described the macroscopically visible estrous cycle of the viviparous Cyprinodont *Lebistes reticulatus*, which is characterized by a typical attitude, the "elevation-position" (Figs. 87 and 88). At 28° C the cycle lasts from 4 to 6 days. JASKI succeeded in closely analysing the rhythm of the elevation-positions by the daily measurement of the

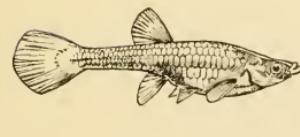


Fig. 87. Virgin female *Lebistes* without elevation; above: swimming; below: in rest. (Thesis JASKI).

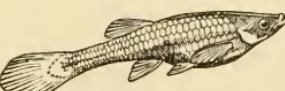


Fig. 88. Virgin *Lebistes* females with elevation. Above: swimming; centre and below: two animals with different elevation, in rest (JASKI).



angle between the body axis of the fish and the water-level. By plotting the angular increase and decrease against time, the Figures 89 and 90 have been produced.

JASKI distinguishes autonomous and heteronomous elevations. The former result from endocrine activity in the female, the latter through the action of the male.

In virgin fishes and in the absence of any males, individual estrous cycles occur. From the moment males are brought together with such females the cycles are co-ordinated. When

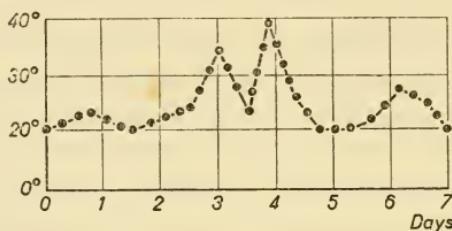


Fig. 89. Elevation curves. The time is plotted horizontally; vertically: the angle of inclination of the part of the spinal column between the swimming-bladder and the anal fin, the angle being determined at least four times a day. The figure shows the average for 20 females after administration of copulin, the autonomous cycle not being suppressed.

The copulin was that of males kept in another aquarium. (JASKI).

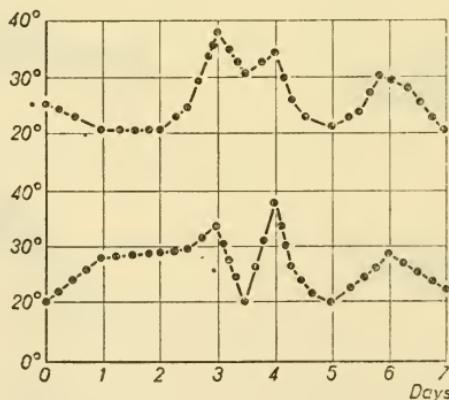


Fig. 90. Elevation curves of two different animals from the experiment of Fig. 89. These two curves show the greatest deviation from the average (JASKI).

virgin females were placed in water which had previously contained males, the same thing happened. Evidently some substance is secreted by the males, which stimulates the elevation-position in the females.

The water in which *Lebistes* males have been swimming causes growth of the ovipositor in the female bitterling. This is also true of the water which has contained male bitterlings (Fig. 91). JASKI calls the active substance secreted by these male fishes, *copulin*.

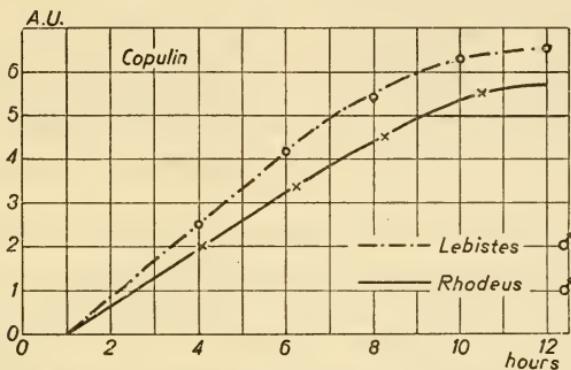


Fig. 91. Ovipositor growth-curves obtained in water activated by *Lebistes* and *Rhodeus* males, respectively.

Following JASKI's investigations, BRETSCHNEIDER studied the cycle in the pregnant female. In *Lebistes*, the oocytes, fertilised in the follicle, develop there into young fishes. Pregnancy lasts about 28 days, and is generally soon followed by another. *Lebistes*, therefore, is a poly-estrous fish. Since the sperm of one fertilisation serves for more than one pregnancy it was not possible to ascertain precisely the moment of fertilisation, for which reason the development of the embryos was used as time-determinant.

The sperm penetrates the pre-formed stigma of the follicle and enters the mature oocyte, fertilising the latter inside the follicle (Fig. 92). The number of mature oocytes thereby falls to zero; but when pregnancy is only half completed, medium-sized oocytes begin to mature and are ready to be fertilised when pregnancy is completed. The developing ovum is supplied by a rich capillary system in the follicular

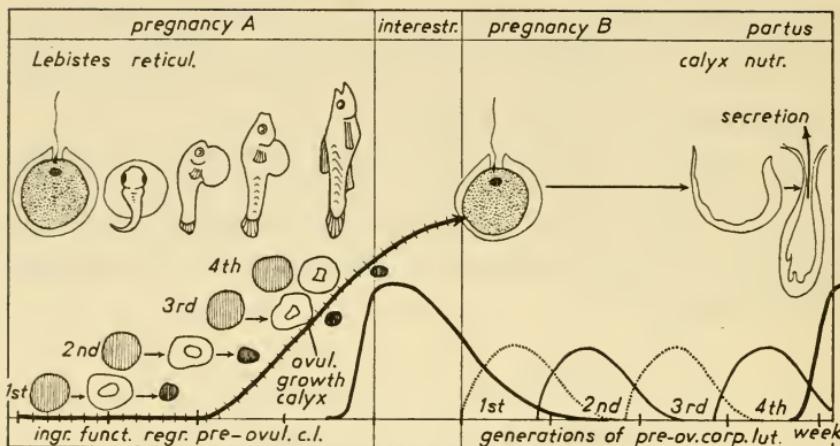


Fig. 92. Cyclic changes in the pregnant *Lebistes* female.

wall and the wall of the yolk-sac. In addition to the intake of oxygen and the loss of carbon dioxide there is also assimilation of water and of salts. The embryo is accordingly heavier than the fertilised ovum. We do not yet know whether interchange of organic substances occurs. While the foetus is still developing, the follicle ruptures where the follicle-stigma was located, so that the lumen of the follicle and that of the ovary become connected. The young fishes leave the follicle through this opening, the follicle remaining behind as calyx. Then, for some time, the granulosa secretes an embryotrophe, which nourishes the young fishes in the lumen of the ovary. During this brief period, therefore, the calyx nutritius, in its secretion of an embryotrophe, behaves as the calyx nutritius in *Zoarces*.

After parturition, these calyces involute; they persist until half-way through the next pregnancy, after which they appear only as scars. Pre-ovulation corpora lutea are found in *Lebistes*, as in *Zoarces*, during the entire course of pregnancy, in at least 4 generations, and preliminary, functional and regression-stages exist side by side. The intensive luteinisation and copious blood supply of these corporea lutea point to considerable activity, of the same nature as in *Zoarces*, which in virgin females runs parallel with their

elevation-state and is comparable with the corpus luteum effect in pregnant females. The time of maximum elevation and that of the maximum number of pre-ovulation corpora lutea coincide (Fig. 93). No doubt, oviductin acts on the follicles, the ovary wall and the genital aperture, causing hypertrophy of the ovary, and distention of the oviduct and genital aperture, resulting in the elevation-position, which provides the conditions necessary for copulation. This elevation-cycle may repeat itself several times. It precedes fertilisation and is completed in pro-estrus. The synchronisation of the elevation-position and the formation of corpora lutea is not necessarily due to any direct connexion between the two, but may result from various causes. JASKI assumed that, through the resorption of large ova and growth of smaller ova, a shift of the centre of gravity of the body took place, which he supposed to cause elevation. This mechanical explanation, however, proved to be incorrect, as the follicular derivatives, described by him as being "transformed follicles", were merely calyces of primiparous females.

To summarize: In the *Lebistes* female, both preparation for pregnancy and pregnancy itself are accompanied by the formation pre-ovulation corpora lutea; the calyces nutriciae secrete embryotrophe, and in pro-estrus, the elevation cycle alternates with the pregnancy cycle.

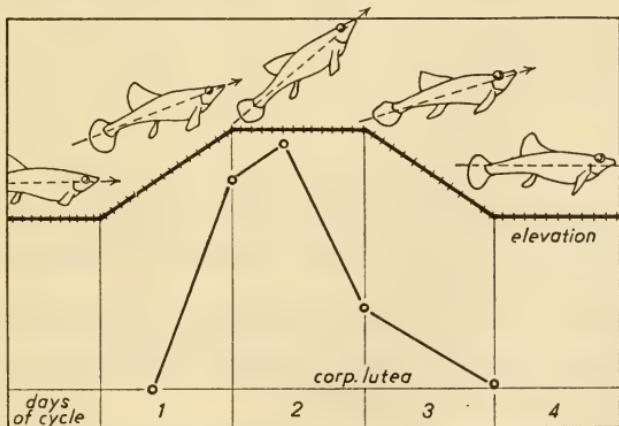


Fig. 93. Correlation between elevation and corpus luteum formation in *Lebistes reticulatus*.

§ 4. THE SEXUAL CYCLE OF *BUFO BUFO* ♂ AND ♀

Among Amphibia, as second group of Anamnia, *Bufo bufo* interested us, especially because of the organ of BIDDER, which, in the adult animal is present in both sexes, and takes an active part in the sexual cycle. The statistical histological investigation made by BRETSCHNEIDER extends to the 5th year of life in this species of toad, and relates to both males and females. So far the following parts of the genital apparatus have been examined.

a. *The fat-body* lies in front of the gonad, and its behaviour during the reproductive cycle is similar to that of other organs associated with metabolism. The only significant fact is that the boundary between the fat-body and the organ of BIDDER is variable.

b. *The organ of BIDDER* lies behind the fat-body and may be regarded, in both sexes, as a rudimentary ovary which, however, shows certain cyclic variations. In both sexes the germinal tissue produces oocytes which, together with their follicular wall, form genuine corpora lutea, whose secretion acts upon the oviduct. The formation of corpora lutea does not differ in any respect from that in *Rhodeus*, but is limited to fairly young oocytes, which remain undeveloped. Those exceptions in which a few BIDDER-oocytes continue to grow to considerable size show the ovarian nature of this organ, and support the supposition that it actually is a rudimentary ovary.

c. *The ovary* lies behind the organ of BIDDER. It forms oocytes, some of which change into corpora lutea while others mature and ovulate, the ruptured follicle remaining behind as calyx simplex.

Oogenesis and corpus luteum formation in the ovary were examined cytologically, and compared with similar processes occurring in the organ of BIDDER.

d. *The testis* arises from the genital tract. Its development, spermatogenesis and the interstitium were examined cyto-

logically and statistically. The influence of gonadotrophic mammalian hormones upon these tissues was also studied experimentally.

e. *The oviduct* is present in both sexes. In the female it is a tract rich in "shell"-glands, while, in the male, it is a rudimentary tract without any established function, but structurally similar to its gonad, the organ of BIDDER.

Although an extensive literature already exists on the organ of BIDDER, we still lack a wider and more comprehensive survey of the entire sexual-endocrine organization of *Bufo bufo*. We believe we can do this by regarding the organ of BIDDER in both sexes as a potential ovary, forming pre-ovulation corpora lutea whose hormone serves to stimulate the oviduct and keep it at the required size and degree of sensitivity.

Viewed in this light, the sexual-endocrine organization of the female has (a) a potentially female half consisting of a rudimentary ovary and the organ of BIDDER, which forms corpora lutea whose hormone brings the oviduct into the pre-mature state, and (b) a genuinely female half consisting of an ovary forming both mature oocytes and pre-ovulation corpora lutea, whose secretion brings the oviduct from the pre-mature into the estrous stage.

Similarly, the sexual-endocrine organization of the male has (a) a potentially female half, with a potential ovary, the organ of BIDDER, and a potential oviduct influenced by the corpus luteum hormone from the organ of BIDDER; and (b) a genuinely male half consisting of the testis and the vasa deferentia. After removal of the testis, the organ of BIDDER develops to form an ovary, and the potential oviduct becomes a true oviduct. Thus the original male is changed into a female. In support of this interpretation we may briefly cite the following arguments:—

f. *The significance of the organ of BIDDER and the ovary in the female.*

The weight of the organ of BIDDER reaches a maximum in the spring preceding the first ovulation (Fig. 94 A), and

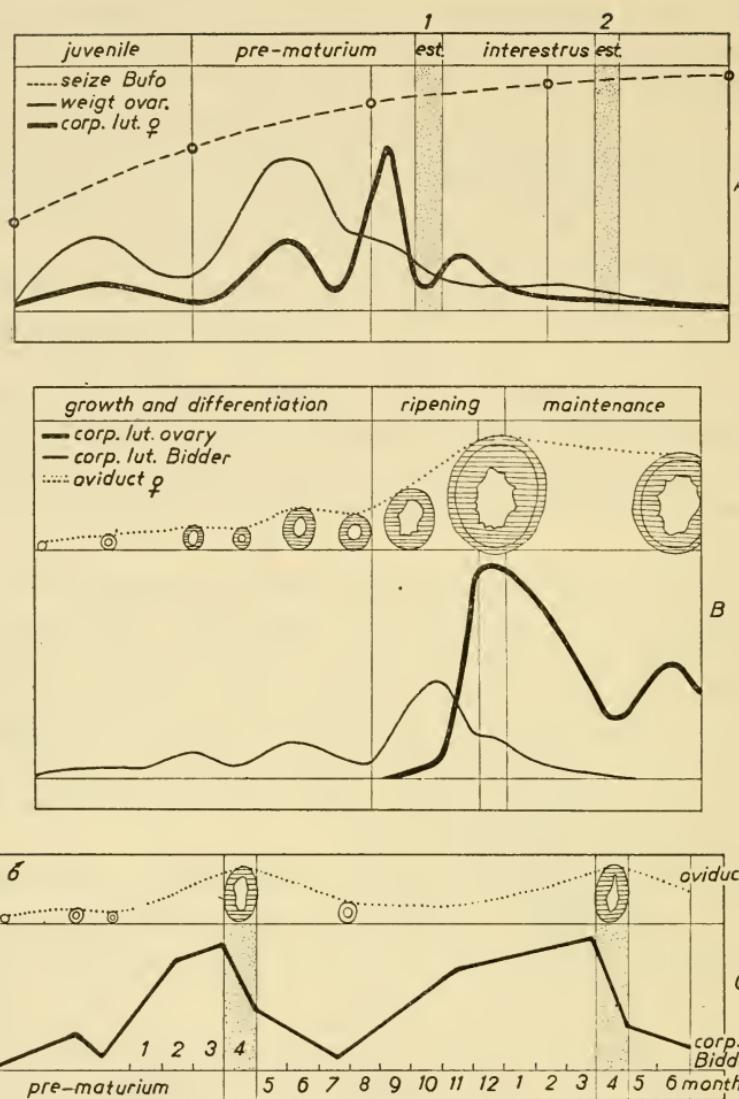


Fig. 94. Correlation between corpus luteum formation in the ovary and in the organ of BIDDER, and development of the oviduct in *Bufo bufo* during the sexual cycle.

falls rapidly thereafter, so that, in older animals, it may be either rudimentary or absent. Parallel with this is the production of corpora lutea in this potential ovary, which reaches a maximum just before maturity (Fig. 94 A), after

which it drops again. In comparison to the size of the animal as indicating its age, we may say that the significance of the organ of BIDDER is inversely proportional to the age of the animal. If we compare the development of the oviduct up to the first oviposition with the production of corpora lutea from both the potential and the genuine ovary (Fig. 94 B), we see that growth and shrinkage of the oviduct run parallel with the frequency of corpora lutea. In the young animal in the summer, there are a great number of corpora lutea, and the oviduct is large; but in winter it is small. While the organ of BIDDER is active, in pro-estrus, the oviduct expands strongly and at estrus numerous large ovarian corpora lutea appear. The decreasing hormone content of the involuting organ of BIDDER is compensated by the enormously increasing hormone content of the ovary. During the inter-estrus which follows, the ovarian corpora lutea regulate the size of the oviduct and prevent its atrophy, which would set in on castration (Fig. 94 B). During the period of growth and differentiation the oviduct is influenced by the corpora lutea of the organ of BIDDER, whose incration prepares it for the subsequent functional period. It hypertrophies during the pro-estrus under the influence of the corpora lutea in the organ of BIDDER and the ovary, and is maintained, during inter-estrus, at a sufficient degree of development by the corpora lutea of the ovary to prevent atrophy.

g. *The significance of the organ of BIDDER in the male.*

The possession of a potential ovary and oviduct suggests that the male *Bufo* is hermaphrodite. Whereas, in many amphibia, the rudimentary oviduct atrophies, in *Bufo bufo* it remains quiescent. It is largest in size simultaneously with a maximum production of corpora lutea in the organ of BIDDER, and smallest in the inter-estrus (Fig. 95), as in the female. Neither our own histological analysis nor the experimental investigations of other writers were able to prove convincingly the existence of any other function of the organ of BIDDER in the male. We can explain the significance of

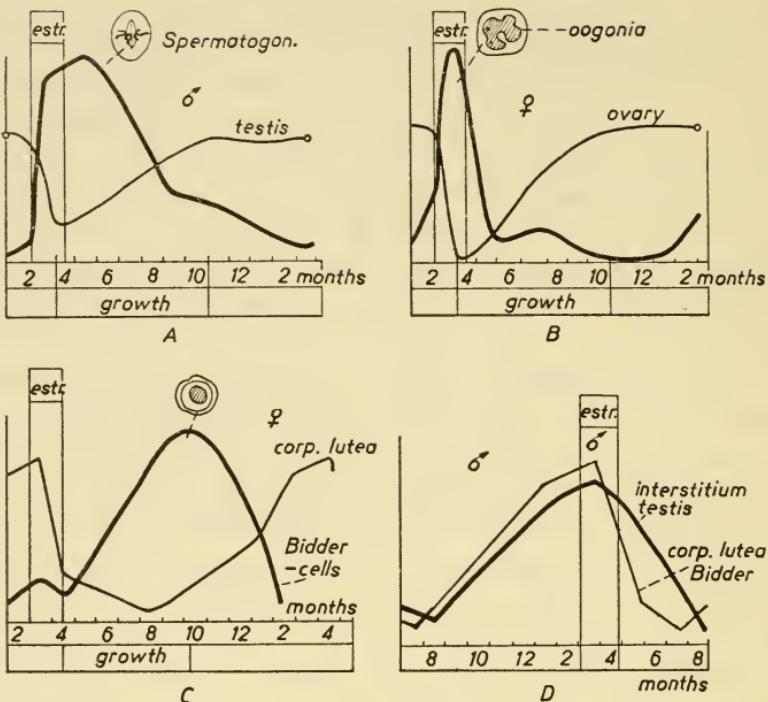


Fig. 95. Relation between the development of spermatogones, testis and the organ of BIDDER in *Bufo bufo* during the cycle.

the organ of BIDDER and the oviduct in the sexual-endocrine organization of the male toad by assuming that the rudimentary ovary and its accessory oviduct constitute a unit, which behaves independently of its possessor determined as being a male; a unit, therefore, which forms corpora lutea in the organ of BIDDER, and, as an oviduct, reacts to the hormone thereof. The organ of BIDDER and the male oviduct may be regarded as vestiges rather than as an integral functional part of the sexual-endocrine system. (See experiments of WITSCHI and others on BIDDER'S organ and sexual differentiation of Amphibia).

i. *The pituitary influence on the sexual-endocrine organization of *Bufo bufo*.*

Both in the testis and in the genuine ovary the greatest emission of gametes takes place during the breeding season.

Reciprocally with this loss, there is an intensive activity in the germinative tissue, resulting in the formation of spermatogonia and oogonia (Fig. 96 A, B). The organ of BIDDER is no exception, for there, too, restitution is greatest in the summer months following the breeding period (Fig. 96 C).

The size of this organ diminishes in winter with the formation of corpora lutea, and increases again in summer. It is probable that the gametogenesis is prompted by a gonadotrophic stimulus from the hypophysis (Fig. 96). It is also evident from the atrophy of the organ of BIDDER, and the gonads after hypophysectomy (HOUSSAY, 1929) that this potential ovary is subject to a similar gonadotrophic influence from the hypophysis.

Experiments on mammals with gonadotrophic hormones such as Ambinon (anterior pituitary extract "Organon") and Pregnyl (chorionic gonadotrophin "Organon") showed two different effects in the male. Ambinon caused, through some luteinisation factor, a marked increase of corpora lutea in

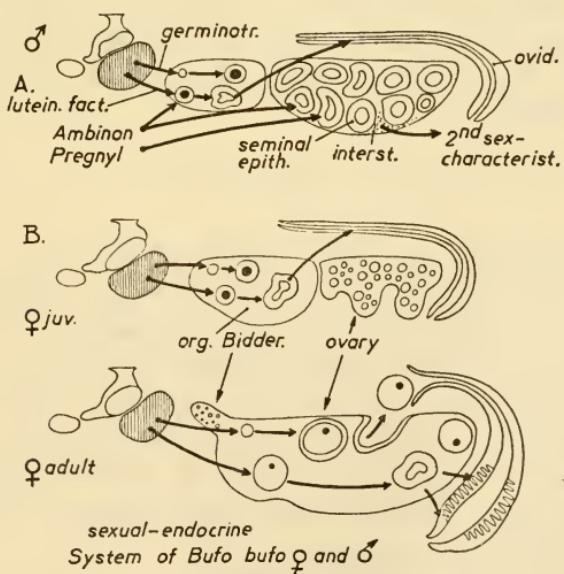


Fig. 96. The pituitary influence on the sexual endocrine organization in *Bufo bufo*.

the organ of BIDDER. In the testis tubules it acts both on the spermatocytes, which differentiate rapidly, and on the SERTOLI-cells, by freeing strands of sperm on a large scale. The loose, differentiated spermatozoa thereby arrive in the lumen of the vessel, filling at the same time the vasa efferentia, the kidney tracts and the vas deferens with sperm.

Pregnyl, on the other hand, caused differentiation and freeing of spermatozoa only in the testis, but had no effect on the organ of BIDDER (Fig. 94).

Two or more factors present in Ambinon, act on the epithelium; one upon the granulosa epithelium and the oocyte; the other on the SERTOLI- and seminal epithelium.

The growth of the interstitial tissue in the testis which, in view of its origin, may be compared to the theca of a follicle, runs almost parallel with the frequency of corpora lutea in the organ of BIDDER. It reaches its maximum at breeding period and its minimum in summer (Fig. 93 D). It is suggested that the hormone of the interstitial cells influences the sexual characteristics of the male. The corpus luteum hormone stimulates the oviduct as adnex to the female part of the sexual apparatus, and the testis hormone stimulates the secondary sexual characteristics as adnexa of the male part.

### § 5. SUMMARY

To summarize we have concluded that the organ of BIDDER, as a potential ovary in the female maintains and prepares the oviduct until the first oviposition. It atrophies, however, as soon as the ovary, together with its pre-ovulation corpora lutea, takes over this function (Fig. 93 B). Some of the large follicles change into corpora lutea, whose hormone stimulates the wall of the oviduct to secrete the egg-shells. The rest of the follicles mature and ovulate; the eggs are laid, and the calyces remain.

In the male, the organ of BIDDER, as a potential ovary, and the oviduct do not play an active part in the sexual-endocrine organization; but by virtue of their vestigial struc-

ture they are independent of the testis and of the secondary sexual characteristics. This is expressed in the formation of corpora lutea and in the response thereto on the part of the oviduct. Under the influence of the same pituitary factors, the cycle of both the gonad and the organ of BIDDER temporarily coincide. The organ of BIDDER and the oviduct maintain themselves in the male, after the first estrus, while in the female they are maintained, after the first estrus, by the corpus luteum hormone of the ovary.



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## SUBJECTS INDEX

	Page		
Activity of adrenal cortex extracts . . . . .	28	Corpus luteum, degeneration	90
— — androstanes . . . . .	25	— — histogenesis . . . . .	81
— — corpus luteum extracts . . . . .	30	— — pre- and post-ovulation . . . . .	93 98 100
— — glandular extracts . . . . .	26	— — progesterone content	30
— — hormone preparations (definition) . . . . .	15	— — solidum . . . . .	104
— — ovary extracts . . . . .	33	Embryotrophe . . . . .	128 133
— — pregnanes . . . . .	15 16	Fat body in <i>Bufo bufo</i> . . . . .	134
— — testis extracts . . . . .	27	Follicle, definition . . . . .	81
— — urine . . . . .	34	— — genesis of . . . . .	79 80
Anal fin unit (A.U.), definition . . . . .	5	— — potentialities . . . . .	96
Atresia . . . . .	98	— — theca externa of . . . . .	79
Atretic cyst . . . . .	103	Folliculus atreticus cavus . . . . .	102
Bitterling as testobject . . . . .	4	— cavus . . . . .	95
— biology . . . . .	1	— solidus . . . . .	95
— diet . . . . .	6	Hormone preparations, see	
— ovary . . . . .	77	Activity	
— oviposition . . . . .	120	Hypophysis, see pituitary	
— ovipositor . . . . .	2	Interstitial . . . . .	34 108
— sensitizing . . . . .	5	<i>Lebistes reticulatus</i> . . . . .	129
— sexual behavior during spawning		Lobus intermedius . . . . .	61
— sexual cycle . . . . .	117	— posterior . . . . .	61
<i>Bufo bufo</i> . . . . .	134	— tuberalis . . . . .	60
Calyx . . . . .	100	Lutein cyst . . . . .	98
— cyst . . . . .	107	Mussel . . . . .	123
— mixtus . . . . .	107	Organ of Bidder . . . . .	134 135 137
— nutricius simplex . . . . .	107	Ovary of bitterling . . . . .	77
— simplex . . . . .	106	Oviductin . . . . .	56 109
— with retention of ovum		Oviposition of bitterling . . . . .	120
Copulin . . . . .	131	Ovipositor as a pregnancy test . . . . .	36
Corpus albicans . . . . .	108	Ovipositor growth caused by <i>allo-pregnandiol</i> . . . . .	13
Corpus atreticum mixtum . . . . .	102	— — — — <i>allo-pregnandione</i> . . . . .	11
— — ruptum . . . . .	103	— — — — <i>allo-pregnanolone</i> . . . . .	12
— — solidum . . . . .	102	— — — — androstane-	
— — with retention of ovum . . . . .	103	diol . . . . .	24
Corpus candidans . . . . .	108	— — — — androstane-	
		dione . . . . .	20
		— — — — androstene-	
		diol . . . . .	19

	Page	
— — — androstenedione . . . . .	18	Ovipositor growth during spawning time . . . . .
— — — cis-androsterone . . . . .	22	— — mechanism of erection . . . . .
— — — trans-androsterone . . . . .	22	— — its significance in endocrinology . . . . .
— — — corticosterone . . . . .	22	— — measurement . . . . .
— — — cortine . . . . .	16	— — test, specificity . . . . .
— — — trans-dehydroandrosterone . . . . .	29	Ovo-follicular system . . . . .
— — — cis-dihydro-testosterone . . . . .	19	Ovum, conversion into corpus luteum . . . . .
— — — trans-dihydrotestosterone . . . . .	23	— — phagocytosis of yolk . . . . .
— — — desoxycorticosterone . . . . .	24	Pituitary, acidophilia and basophilia . . . . .
— — — desoxycorticosteroneacetate . . . . .	16	— — and corpus luteum production . . . . .
— — — equilenin . . . . .	18	— — blood-supply . . . . .
— — — estradiol . . . . .	9	— — gonadotrophic zone . . . . .
— — — estriol . . . . .	9	— — haemo- and neurocrinia . . . . .
— — — estrone . . . . .	9	— — homology of lobes . . . . .
— — — methyltestosterone . . . . .	9	— — hormone transport . . . . .
— — — pituitary extracts . . . . .	24	— — innervation . . . . .
— — — pregnane-diol . . . . .	46	— — light and activity . . . . .
— — — pregnane-dione . . . . .	14	— — platy- and leptobasia . . . . .
— — — pregnanalone . . . . .	14	Pre-ovulation corpus luteum
— — — pregnenedione . . . . .	14	cavum . . . . .
— — — pregnenolone . . . . .	13	— — ruptum . . . . .
— — — progesterone . . . . .	14	— — with retention of ovum . . . . .
— — — solvents . . . . .	10	— — — cavum . . . . .
— — — trans-testosterone . . . . .	7	— — — mixtum . . . . .
— — — testosterone propionate . . . . .	19	— — — solidum . . . . .
— — — vitamin E . . . . .	24	— — — with temporary retention of ovum . . . . .
	8	Rhodeus amarus, see bitterling.
		Sexual behaviour of bitterling during spawning
		123 124
		Sexual cycle of <i>Bufo bufo</i>
		134
		— — — <i>Lebistes reticulatus</i>
		129
		— — — <i>Rhodeus amarus</i>
		117
		— — — <i>Zoarces viviparus</i>
		126
		<i>Zoarces viviparus</i>
		126

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RESEARCH IN HOLLAND  
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